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STUDIES ON THE BIOASSAY OF HORMONES

THE ASSAY OF TESTOSTERONE PROPIONATE AND ANDROSTERONE BY A CHICK COMB INUNCTION METHOD[1]

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PREVIOUSLY Frank et al. (1942) described a chick comb method for the assay of androgens. This communication is concerned with the application of this method to the assay of androsterone and testosterone propionate.

ANIMALS, METHODS, MATERIALS

White Leghorn male and female chicks obtained from the Kerr Chickeries, Frenchtown, New Jersey were employed in this study. They were shipped from the hatchery on the day of hatching and received in Cleveland the following day at the age of 1 to 2 days. The following day, at the age of 2 to 3 days, inunctions were started and continued once daily for 7 days. The total dose of androgens was applied to the comb in a total of 0.35 cc. of corn oil. The assays were carried out as described in detail by Frank et al. (1942).

The androsterone and testosterone propionate were kindly supplied by Ciba Pharmaceutical Products, Inc.

EXPERIMENTAL

Assay of Androsterone

Androsterone was assayed at levels of 10 to 40 micrograms and calculated according to the formulations of Frank et al. (1942). The

results of 20 experiments and 6 groups of controls are represented in Table I. When the formula of Frank et al. (1942) was applied to the

TABLE I. THE ASSAY OF ANDROSTERONE BY THE METHOD
OF FRANK ET AL. (1942)

Androsterone administered micrograms	Number of chicks used		Amount of androsterone recovered micrograms	Error %
	Males	Females		
0	9	11	4.4	—
0	7	13	4.2	—
0	8	11	6.4	—
0	8	11	6.0	—
0	5	12	6.0	—
0	11	8	4.4	—
10	14	5	16.9	+69
10	7	9	20.0	+100
15	8	11	8.2	−45
15	4	10	20.0	+33
20	10	9	18.4	− 8
20	9	11	22.0	+10
20	11	6	22.0	+10
20	13	7	21.2	+ 6
20	11	6	22.1	+11
20	7	10	23.4	+17
20	9	8	20.4	+ 2
20	6	13	19.5	− 3
25	11	9	24.4	− 2
30	11	9	27.3	− 9
30	14	5	21.3	−29
30	6	13	23.2	−23
30	8	10	33.3	+11
40	5	15	29.2	−27
40	7	13	32.3	−19
40	7	13	26.8	−33

controls, the values indicated a mean of 5.2 mg. of androsterone with a variation from 4.2 to 6.4 mg. for the individual groups. At the lowest dose studied, 10 mg., an overestimate was indicated with errors of +69 and +100 respectively while at the highest dosage level, 40 mg., there was an underestimate in the order of 19 to 33 per. cent. Between the levels of 15 to 30 micrograms of androsterone there was a tendency for both positive and negative errors.

Assay of Testosterone Propionate

For the assay of testosterone propionate the essential details of the method were preserved but the initial body weights of the chicks were not considered. The final comb weights and the body weights were determined and the ratio of the comb weight in milligrams to the body weight in grams was used for the calculations. Both male and female chicks were employed.

Table II illustrates the responses to testosterone propionate from 5 mg. to 160 mg. All the data contained in this table were determined during one run. In order to determine the predictable error of the method one-half of the observations are considered as the standard

TABLE II. THE RESPONSE OF THE CHICK COMB
TO TESTOSTERONE PROPIONATE
(Response expressed as ratio of comb weight in
milligrams to body weight in grams)

5µg.		10µg.		20µg.		40µg.		80µg.		160µg.	
S♂	U♂	S♂	U♂	S♂	U♂	S♂	U♂	S♂	U♂	S♂	U♂
0.45	0.48	0.38	0.98	0.44	0.51	1.10	0.96	1.33	1.13	2.46	2.16
0.51	0.52	0.56	0.73	0.58	0.69	0.67	1.10	1.10	1.31	1.62	1.38
0.63	0.68	0.53	0.84	1.13	0.50	0.81	0.80	0.97	1.30	1.60	2.71
0.67	0.50	0.75	0.71	1.02	0.56	0.81	0.89	1.17	0.77	1.30	3.21
0.75	0.62	0.66	0.67	0.58	0.41	1.37	1.52	1.64	1.34	1.15	1.13
0.48	0.67	0.66	0.53	0.55	0.59	0.90	0.61	1.55	1.48	1.25	2.00
0.69	0.59	0.42	0.68	0.82	0.69	0.71	0.45	1.11	1.46	1.72	1.17
0.50	0.47	0.77	0.56	0.76	0.44	0.63	1.08	1.48	1.11	1.23	1.91
S♀	U♀	S♀	U♀	S♀	U♀	S♀	U♀	S♀	U♀	S♀	U♀
0.43	0.37	0.43	0.61	0.63	0.83	0.36	0.64	0.76	1.15	0.85	1.53
0.32	0.32	0.38	0.41	0.68	0.84	1.68	0.69	0.92	1.42	2.03	1.09
0.53	0.58	0.54	0.55	0.57	0.69	0.46	0.93	0.96	0.88	2.65	1.63
0.37	0.67	0.56	0.55	0.55	0.73	0.69	0.89	0.84	0.51	0.89	1.60
0.45	0.62	0.80	0.48	0.92	0.82	0.78	0.73	0.82	1.16	2.12	2.05
0.39	0.37	0.43	0.36	0.66	0.51	0.84	1.69	1.34	1.20	2.76	2.66
0.67	0.49	0.51	0.49	0.58	0.43	0.82	0.97	0.96	1.13	1.59	1.98
0.47	0.38	0.49	0.42	0.65	0.53	0.86	0.76	0.76	1.21	1.14	1.90

and the remaining half of the observations as those belonging to the unknown. This is true both for the data on the males and the females. Thus the dosage for both the so-called standard and unknown are the same. In a preliminary analysis it was found that when the data were plotted as a log dose-response relationship an exponential function was apparent. When reconsidered as a log dose-log response relationship a straight line function was evident. Therefore, this log dose-log response was used for further analysis of the data.

These data (Table II) were subjected to analysis by the statistical methods of Bliss (1944), Irwin (1937), and Bülbring (1935). Table III illustrates the predictable error when analyzed by the method of Irwin and Bülbring. By this method 3 dose levels of both the standard and unknown were employed. At each dose level 16 animals (8 males and 8 females) were employed for both the standard and the unknown. Thus under these conditions a total of 96 animals was employed. At the dosage levels of 5, 10, and 20 micrograms an error range of -45 to $+84$ per cent was found at $P = 0.95$. When the same number of groups of animals were employed at the range of 20 to 160 micrograms the error range was considerably reduced. At 20, 40, and 80 micrograms a range of -24 to $+32$ per cent was found while at the levels of 40,

TABLE III. RELATIVE POTENCIES AND ERRORS BY
THE METHOD OF IRWIN

(Relationship between log dose and log response)

Number of chicks in each group		Concentration of testosterone propionate used μg.	Ratio of potency standard / Unknown	Errors P = 0.95	
Males	Females			Low %	High %
8	8	5, 10, 20	$\dfrac{100}{90.6}$	45	84
8	8	20, 40, 80	$\dfrac{100}{101}$	24	32
4	4	20, 40, 80	$\dfrac{100}{117}$	43	73
8	8	40, 80, 160	$\dfrac{100}{118}$	17	21

80, and 160 micrograms the error range at $P = 0.95$ was -17 to $+21$ per cent.

When a total of 48 animals was employed instead of 96 animals the error range increased considerably from -24 and $+32$ per cent to -43 and $+73$ per cent.

In Table IV the data are considered by the method of Bliss (1944). Here 2 dose levels of both the standard and the unknown were employed and the number of animals in each group is 16 (8 males and 8 females) in three comparisons and 12 (6 males and 6 females) in two comparisons.

TABLE IV. RELATIVE POTENCIES AND ERRORS BY
THE METHOD OF BLISS

(Relationship between log dose and log response)

$$\frac{\text{High Dose of Standard}}{\text{Low Dose of Standard}} = \frac{\text{High Dose of Unknown}}{\text{Low Dose of Unknown}}$$

Number of chicks		Concentrations of testosterone propionate used μg.	$M \pm S_m$	Error P = 0.95	
Males	Females			Low %	High %
8	8	40, 160	128 ± 20.7	27	38
6	6	40, 160	129 ± 24.3	31	46
8	8	20, 80	87 ± 14.4	27	38
6	6	20, 80	95 ± 20.4	35	54
8	8	20, 40	102 ± 22.6	38	56

When 12 animals were employed per group a greater error of the potency ratio was found than when 16 animals were used but the difference was not particularly striking. At the levels of 40 and 160 micrograms groups of 16 (total animals was 64) an error range of −28 and +38 per cent was found as compared to an error range of −31 and +46 per cent for groups of 12 (total animals was 48). In a second instance where groups of 16 and 12 were compared at the 20 and 89 microgram levels, it was found that an error range of −27 to +38 per cent was found for the groups of 16 while the groups of 12 showed an error range of −35 to +54 per cent.

The highest error range of the potency ratio was found at the levels of 20 and 40 micrograms in spite of the fact that 16 animals were employed in each group. This range of error was from −38 to +56 per cent.

No significant difference was found in the slopes of the unknown and standard by either method of calculation.

DISCUSSION AND CONCLUSION

The data presented in this report confirm the findings of Klemper et al. (1942) as to the accuracy of the assay of androsterone. These workers reported a mean error of 13 per cent for 24 determinations of androsterone between 20 and 40 micrograms. For 16 determinations of androsterone between 20 and 40 micrograms we have found a mean error of 12 per cent. For 39 determinations between 10 and 50 micrograms these workers found a mean error of 24.6 per cent, while for the range 10 to 40 micrograms we have found a mean error of 24 per cent.

Still to be determined is the adaptability of this method to the assay of other androgens in terms of androsterone.

The method for the assay of testosterone propionate as presented in this paper appears to be of value both from the standpoint of sensitivity and reproducibility. Within the range of 20 to 160 micrograms the use of 48 animals on the unknown run in parallel with 48 animals on the standard accuracies of better than 32 per cent in potency ratio ($P = 0.95$) can be realized. With the use of 32 animals on the standard and 32 chicks on the unknown run simultaneously, errors in potency ratio better than 38 per cent ($P = 0.95$) can be realized between the dosage range of 20 to 160 micrograms. Recently Mathieson and Hays (1945) have demonstrated the feasibility of assaying testosterone propionate in castrate male rats using the experimental design of Bliss (1944). The expected error of the potency ratio using a total of 32 rats was similar to the error range found using 64 chicks.

SUMMARY

The utility of the Frank et al. (1942) chick comb method for the assay of androsterone has been confirmed. Using 16 to 20 chicks per

group and working in the range of 20 to 40 micrograms of andros-
terone a mean experimental error of 13 per cent was found.

A method is described for the assay of testosterone propionate in
oil solution. The method employs the essential details described by
Frank et al. (1942) except that the experimental design was changed
so that the unknown was run in parallel with the standard. Within
the range of 40 to 160 micrograms of testosterone propionate and
with the use of a total of 96 chicks an accuracy of -17 to $+21$ per
cent ($P = 0.95$) in the determination of the potency ratio was realized.

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STUDIES ON THE BIOASSAY OF HORMONES
THE RELATIVE REACTIVITY OF THE COMB OF VARIOUS BREEDS OF CHICKS TO ANDROGENS[1]

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It HAS been known for some time that various breeds of fowls differ as to their practicability for use in androgen assays when the comb is used as the test organ. Thus the White and Brown Leghorns and the English Game Cock have been used on the basis of their relatively high sensitivity to androgens. The interest in the difference in comb reactivity goes beyond the question of utility of various breeds in the androgen assay. The reasons for the differences in the reactivity may serve as a valuable clue to the factors which operate in the reactivity of the comb to androgens as well as the mechanism of action of the androgens on the comb.

This communication deals with experiments on the reactivity of the combs in various species of fowls to androgens when the hormone was administered by direct application to the comb. This procedure should rule out the question of the endogenous material and the metabolism or inactivation of the hormone in the body. In these experiments we are dealing only with the direct stimulation and the local inactivation of the hormone at the site of the comb.

ANIMALS, MATERIALS, METHODS

The three breeds of chicks, the White Leghorn, the Rhode Island Red, and the Barred Rock, were obtained from the Kerr Chickeries, Frenchtown, N. J., and were received in Cleveland one to two days after hatching. The animals were kept in thermostatically controlled brooders and fed chick starting mash and water exclusively. The chicks were 2 to 3 days of age at the start of the experiment. The total dose of testosterone propionate[2] was contained in 0.35 cc. of corn

oil and administered once daily for seven days. Five-hundredths of a cc. of the hormone solution was dropped on the comb from a 1 cc. tuberculin syringe fritted with a number 24 hypodermic needle. Twenty-four hours after the last hormone application, the animals were sacrificed and body weight and comb weights were determined. The animals were autopsied at 9 to 10 days of age. The comb responses are expressed as the ratio of the comb weight in milligrams to the body weight in grams.

Ten logarithmic dosage intervals of testosterone propionate were studied for two breeds of chicks and 14 intervals for the White Leghorns. The dosage range was from 40 μg. to 20.48 mg. per animal total dose in all three breeds.

EXPERIMENTAL

The body weight of the three breeds studied did not vary significantly. The mean body weights for the male White Leghorns, Rhode Island Reds, and Barred Rocks were 62, 61 and 58 grams, respectively, while the mean body weights of the females of the same species were 58, 59 and 57, respectively. The number of animals per group varied from 34 to 49 animals.

Table I presents a summary of the comb responses to varying concentrations of testosterone propionate. The comb size is represented as the ratio of comb weight in milligrams to the body weight in grams. The data have been considered from a number of points of view which may be listed as follows:

(A) Analysis of control ratios of the respective breeds.

(B) Comparative amounts of androgen necessary to produce a statistically significant increase in comb ratio.

(C) Determination of respective slopes for each of the three breeds such that these slopes are not significantly different among themselves. From these data calculate relative amounts of testosterone propionate for various breeds necessary to produce similar rate of increment in comb ratio with increasing dose.

(D) Comparison of maximum slopes for the three breeds.

(E) Comparison of maximum comb ratios attainable by three breeds after testosterone propionate stimulation.

(A) In none of the three breeds do we find a significant difference between the comb sizes of male and female chicks. On the other hand, there is a distinct difference between the sizes of the combs of the White Leghorn and Rhode Island Red chicks. This difference in comb ratio was found for both males and females. Thus, the comb ratio of White Leghorn males was 0.38 ± 0.01 as compared to 0.25 ± 0.01 for the Rhode Island Reds. The female White Leghorn chicks had a comb ratio of 0.36 ± 0.01 while the Rhode Island Reds of the same

sex had a value of 0.23 ± 0.01. An even greater difference has been found between the comb ratios of the White Leghorn and Barred Rock chicks for both the males and females.

TABLE I. THE RELATIVE REACTIVITY OF THE COMBS OF WHITE LEGHORNS, RHODE ISLAND RED, AND BARRED ROCK CHICKS TO TESTOSTERONE PROPIONATE WHEN ADMINISTERED BY DIRECT APPLICATION TO THE COMB

Breed of chick	Amount administered µg.	Number of chicks		Ratio = $\dfrac{\text{Comb in mg.}}{\text{B.W. in g.}} \pm$ S.E.	
		M	F	M	F
White Leghorn	0	62	56	0.38 ± 0.01	0.36 ± 0.01
	2	13	6	0.46 ± 0.04	0.43 ± 0.06
	5	28	34	0.55 ± 0.02	0.47 ± 0.02
	10	32	31	0.63 ± 0.03	0.52 ± 0.02
	20	18	45	0.65 ± 0.04	0.67 ± 0.02
	40	36	51	0.77 ± 0.05	0.69 ± 0.02
	80	46	38	1.03 ± 0.05	0.91 ± 0.05
	160	29	36	1.53 ± 0.10	0.54 ± 0.09
	2,560	25	13	1.74 ± 0.07	1.74 ± 0.15
	5,120	12	12	1.88 ± 0.15	1.98 ± 0.15
	20,480	8	13	1.89 ± 0.22	1.82 ± 0.16
Rhode Island Red	0	47	29	0.25 ± 0.01	0.23 ± 0.01
	40	14	11	0.33 ± 0.01	0.33 ± 0.02
	80	13	11	0.36 ± 0.03	0.40 ± 0.04
	160	9	14	0.48 ± 0.05	0.49 ± 0.05
	640	9	16	0.85 ± 0.10	0.82 ± 0.10
	1,280	15	10	0.91 ± 0.07	0.87 ± 0.09
	2,560	32	21	0.92 ± 0.05	1.03 ± 0.07
	5,120	11	12	1.14 ± 0.07	1.03 ± 0.08
	20,480	—	19	—	1.10 ± 0.08
Barred Rock	0	36	37	0.21 ± 0.01	0.20 ± 0.01
	40	13	10	0.25 ± 0.02	0.22 ± 0.02
	80	12	13	0.23 ± 0.02	0.29 ± 0.02
	160	9	14	0.36 ± 0.04	0.32 ± 0.02
	640	12	17	0.54 ± 0.03	0.55 ± 0.03
	1,280	11	15	0.61 ± 0.02	0.63 ± 0.04
	2,560	24	31	0.85 ± 0.01	0.82 ± 0.04
	5,120	9	15	0.97 ± 0.09	1.07 ± 0.08
	20,480	12	10	1.01 ± 0.11	1.02 ± 0.07

(B) Males of the breeds White Leghorn, Rhode Island Red and Barred Rock, respectively, require 2, 30 and 40 µg. of the steroid for a significant comb stimulation (20 per cent increase). Compared with the White Leghorn male, we may say that the Rhode Island Red male requires 15 times as much hormone, while the Barred Rock required 20 times as much hormone. Similar consideration of the females indicates that the Rhode Island Red requires 10 times as much hormone as the White Leghorn female and that the Barred Rock requires 20 times more testosterone propionate.

It must be remembered that the actual increment in comb ratios are largest for the White Leghorn and smallest for the Barred Rock chicks. Thus at the level of response considered the actual incre-

ments in comb weight represented are 0.07 mg. per gram of body weight for the White Leghorn, 0.05 mg. per gram for the Rhode Island Reds and 0.04 mg. per gram for the Barred Rocks.

(C) The reactivity of three breeds of chicks, as judged by the amount of hormone needed to attain a certain arbitrary slope of response may be compared. The choice of the slopes was made on the basis of the maximum slopes attainable by the Rhode Island Red and Barred Rock chicks. A portion of the response curve of the White Leghorn chicks was then taken which had a slope similar to those of the less reactive breeds. If the slopes are not significantly different then it is possible to get a measure of the relative comb reactivity of the various breeds by measuring the displacement of the various curves.

TABLE II. THE MAXIMUM SLOPES OF COMB RESPONSE ATTAINABLE BY RHODE ISLAND RED AND BARRED ROCK CHICKS
THE WHITE LEGHORN COMB RESPONSE CURVE WAS TAKEN WHERE THE SLOPE WAS NOT SIGNIFICANTLY DIFFERENT FROM THAT OF THE OTHER BREEDS
(LOGARITHM DOSE-RESPONSE)

Breed of chicks	Sex	Dosage levels of testosterone propionate μg.	Total number of animals	Slope $b \pm$S.E.
White Leghorn	M	20; 40; 40	100	0.677 ± 0.107
	F	10; 20; 40; 80	165	0.357 ± 0.023
Rhode Island Red	M	80; 160; 640	31	0.545 ± 0.026
	F	80; 160; 640	41	0.485 ± 0.110
Barred Rock	M	640; 1280; 2560	47	0.537 ± 0.101
	F	640; 1280; 2560; 5120	78	0.484 ± 0.110

Table II illustrates the various dosage levels that were employed to get, first, the maximum slopes for the Rhode Island Red and Barred Rock chicks and, secondly, a range of dosages for the White Leghorn chicks which yielded slopes not significantly different than those of the other two breeds. All considerations were log dose-response relationships. The statistical analysis was done for both male and female chicks.

For the male chicks the slopes considered were 0.677 ± 0.107, 0.545 ± 0.026, and 0.537 ± 0.101 for the White Leghorn, Rhode Island Red, and Barred Rock chicks respectively. For the female chicks the slopes were 0.357 ± 0.023, 0.485 ± 0.110, and 0.484 ± 0.11 for the White Leghorn, Rhode Island Red and Barred Rock chicks respectively.

In Table III the significance of difference between the slopes was tested by the method of Fischer (1934). The slopes of both the Rhode Island Red and Barred Rock chicks were compared to the slope of the

White Leghorn. In no instance for either the response of the male or female chick's combs was it possible to demonstrate a significant difference in the slopes.

The sensitivities of the respective breeds may be represented as percentages of that of the White Leghorn chicks, the latter sensitivity being considered as 100 per cent. The calculations were carried out for both the males and females of each breed by the method of Bülbring (1935). Considering the male White Leghorn chick's comb to have a sensitivity of 100 per cent, the Rhode Island Red chick's comb was found to have a sensitivity of 10 per cent and the combs of

TABLE III. SIGNIFICANCE OF DIFFERENCE BETWEEN
SLOPES LISTED IN TABLE II

Breeds of chicks compared	Sex	Fisher's t	Number of chicks	P
White Leghorn and Rhode Island Red	M	0.898	129	0.3–0.4
	F	0.955	194	0.3–0.4
White Leghorn and Barred Rock	M	0.868	145	0.3–0.4
	F	0.981	241	0.3–0.4

the male Barred Rock a sensitivity of only 1.8 per cent. The comparative comb sensitivity of the females was similar to that found for the males. When the female White Leghorn comb was assigned a sensitivity of 100 per cent the Rhode Island Red chick's comb was found to be 8.9 per cent and the Barred Rock chick's comb was found to be 1.8 per cent. The error of the determinations at $P = 0.95$ was quite low. The maximum error range being -33 to $+48$ per cent and the minimum error range being -19 to $+23$ per cent.

(D) In the preceding analysis it was found that the White Leghorn chick required far less testosterone propionate to attain a slope similar to that of the other two breeds of chicks. Further the slopes for the Rhode Island Red and Barred Rock chicks were the maximal slopes attainable considering at least three dosage levels. However, the White Leghorn logarithm dose-response curve was not the maximum attainable. In Table IV this point is illustrated for both male and female chicks. Since the maximum slopes for the response relationships of the Rhode Island Red and Barred Rock did not differ significantly, only the maximum slope of the latter group was used for comparison with the White Leghorns. The maximum slope for the male White Leghorn logarithm dose-response relationship was 1.215 ± 0.147 as compared to 0.537 ± 0.101 which was found for the maximum slope of the Barred Rock males. The difference in slopes was significant as seen by the Fischer's t value of 3.027 which indicates a value of P below 0.01. The difference in maximum slopes for female logarithm dose-response curve was also significant. The

White Leghorn females had a maximum slope of 1.361 ± 0.100 as compared to a slope of 0.484 ± 0.110 for the Barred Rock females. The t value was 8.240 indicating a P value well below 0.01.

TABLE IV. COMPARISON OF MAXIMUM SLOPES ATTAINED BY REGRESSION OF WHITE LEGHORN AND BARRED ROCK COMBS ON TESTOSTERONE PROPIONATE. (RHODE ISLAND RED MAXIMUM SLOPES NOT SIGNIFICANTLY DIFFERENT FROM THOSE OF BARRED ROCK)

Sex	Maximum White Leghorn slope[1] $\pm$ S.E.	Maximum Barred Rock slope $\pm$ S.E.	Total Number of chicks	t	P
M	1.215 ± 0.147	0.537 ± 0.101	158	3.027	0.01
F	1.361 ± 0.100	0.484 ± 0.110	223	8.240	0.01

[1] Derived from 40, 80, and 160 μg. doses of testosterone propionate.

(E) In Table V another means of evaluating the reactivity of the comb is considered, namely, the maximum size attainable by the combs of the respective breeds at any dose. When considered on the basis of comb size alone or on the magnitude of the comb ratio the White Leghorn breed outranks the other two breeds. But it must be remembered that the White Leghorn chicks start with larger combs.

TABLE V. COMPARATIVE MAXIMUM COMB RATIOS ATTAINED BY THREE BREEDS OF CHICKS AFTER APPLICATION OF TESTOSTERONE PROPIONATE

Breed of chick	Males		Females		Mean increase male and female 0/0
	Control ratio	Maximum increase 0/0	Control ratio	Maximum increase 0/0	
White Leghorn	0.38	396	0.36	415	405
Rhode Island Red	0.25	357	0.23	364	361
Barred Rock	0.21	344	0.20	426	385

If one considers the data on the basis of percentage increment in comb ratio over and above that of the oil treated controls no significant difference among the groups was found. These calculations are presented in Table V. In the male chicks the maximum increment in comb ratios was 395, 357, and 344 per cent respectively for the White Leghorns, Rhode Island Reds, and Barred Rocks. For the female chicks the maximum increase was 415, 364, and 426 per cent respectively for the various breeds in the same order.

DISCUSSION

The data presented in this report define in a quantitative manner the difference in reactivity of combs of various breeds of chicks to

androgens. The White Leghorn comb is by far the most reactive to androgens when evaluated by any of three criteria: (1) the minimal amount of hormone necessary to produce a statistically significant increment in comb weight; (2) the amounts of hormone necessary to produce a certain arbitrary slope of response on a log dose-response basis; and (3) the maximum attainable slope of response again calculated as a log dose-response relationship.

Since the hormone was administered by direct application the difference in reactivity must be considered as a function of the comb only. That is, no peculiar hormone metabolic differences in the organism as such could be responsible for the difference in the comb reactivity. At least the same quantity of the same hormone was available to the comb.

What then are the possible factors for the differences in the comb reactivities among breeds? First, a difference may exist with respect to the permeability of the comb to hormone. Second, an enzyme system may be present in greater abundance in the poorer reactors which tend to inactivate the administered hormone more efficiently. Third, the abundance of enzyme systems which are concerned with comb tissue synthesis and organization may vary significantly. The responsibility for the differences in reactivity may not be a single factor but a combination of factors.

Further work is in order, particularly on the possible quantitative differences in comb enzyme systems to elucidate this problem.

SUMMARY

The quantitative differences in the reactivity of the combs of three breeds of chicks has been studied. The androgen testosterone propionate was applied to the combs in oil. The dosage range investigated was from 2 micrograms to 20,480 micrograms for the White Leghorn male and female chicks and 40 micrograms to 20,480 micrograms for the Rhode Island Red and Barred Rock breeds.

No significant difference was found in the comb ratios of corn oil control of male and female chicks for any of the three breeds studied but a significant difference was found for the control ratios of the three breeds studied. White Leghorn chicks showed the largest combs, the Barred Rocks the smallest, and the combs of the Rhode Island Reds were intermediate. This order of comb size of control chicks was true for both the males and females.

On the basis of the minimal quantity of testosterone propionate to produce a 20 per cent increment in comb ratio the male White Leghorns were 15 times as sensitive as the Rhode Island Reds and 20 times as sensitive as the Barred Rocks. Similarly the female White Leghorn combs were 10 times as sensitive as those of the Rhode Island Reds, and 20 times those of the Barred Rocks.

The comparative sensitivities of the combs of the three breeds

was evaluated by picking portions of the log dose-response curves where the slopes of all three breeds were not significantly different and using the displacement of the curves as another measure of the relative sensitivity of the various breeds. Under these conditions and expressing the sensitivity of the male White Leghorn chick comb as 100 per cent the sensitivity of the Rhode Island Red was found to be 10 per cent and that of the Barred Rock as 1.8 per cent. In the female chicks the relative comb sensitivities was similarly White Leghorn, 100 per cent; Rhode Island Red, 8.9 per cent; and Barred Rock, 1.8 per cent.

A third criterion of sensitivity of the respective combs to androgen was maximum slope attainable using a log dose-response relationship for at least three points. No significant difference in maximum slope was found for the Rhode Island Red and Barred Rock chick combs but the White Leghorns showed a significantly greater slope.

Finally, an evaluation of the maximum percentage increase in comb size by androgen stimulation revealed that no significant difference could be demonstrated among the three breeds for either male or female chicks.

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THE METABOLISM OF SOME CHEMICAL DEGRADATION PRODUCTS OF ESTROGENS: WESTERFELD'S LACTONE, BIS-DEHYDRO-DOISYNOLIC ACID, ESTROLOLACTONE AND β-ESTRADIOL

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THERE IS a large body of evidence that natural estrogens are inactivated in the liver. *In vitro* and *in vivo* studies are in agreement on this. However, there is a remarkable paucity of evidence as to what actually happens to the administered estrogen. A small portion is converted into other estrogens and excreted in the urine (Doisy, et al., 1942; Doisy, 1942; Pincus, 1946). The recovery of estrogenic activity is increased if the urine is boiled with zinc and hydrochloric acid but there still remains a large proportion of the estrogen for which we are unable to account at present (Smith, 1941).

Chemical degradation of estrogens has yielded estrogenically active materials. It was decided to study some of these which were available in an attempt to shed light upon the problem.

MATERIAL AND METHODS

The sodium salt and the 3-methyl ether of bis-dehydro-doisynolic acid were supplied by Dr. C. R. Scholz of Ciba Pharmaceutical Products, Incorporated (Figure 1).

The Westerfeld's lactone acetate was prepared by the peroxide oxidation method of Mather from estrone acetate supplied by Dr. E. L. Foreman of Lakeside Laboratories and by Dr. Scholz. A small sample was also obtained from Dr. Gregory Pincus. Both our preparation and Dr. Pincus' had an uncorrected melting point of 148°–150°C and a mixture of the two had the same melting point.

The β-estradiol was prepared for us by Dr. Martin Hoffman, of McGill University, from crude material supplied by Dr. Erwin Schwenk of the Schering Corporation. The preparation employed in these studies was of high purity and had a melting point of 220°–221°C.

The estrololactone acetate, the structure of which has not been elucidated as yet, was supplied through the courtesy of Drs. Pincus and R. P. Jacobson of the Worcester Foundation for Experimental Biology.

The same methods were employed as in our previous studies on estrogen metabolism (Segaloff, 1943). Young adult female rats, purchased from Maguran Farms, were spayed. They were then primed with 50 μg. of estrone

in 0.1 cc. of peanut oil, injected subcutaneously. The test injection was then given in 0.05 cc. of 5% benzyl alcohol in sesame oil either subcutaneously or intrasplenically with the spleen *in situ*. In an attempt to get positive results with Westerfeld's lactone acetate and β-estradiol, we injected animals with 0.1 cc. of a saturated solution intrasplenically with the spleen *in*

α ESTRADIOL   β ESTRADIOL

SODIUM BIS-DEHYDRO-DOISYNOLATE   3 METHYL ETHER OF BIS-DEHYDRO-DOISYNOLIC ACID

WESTERFELD'S LACTONE
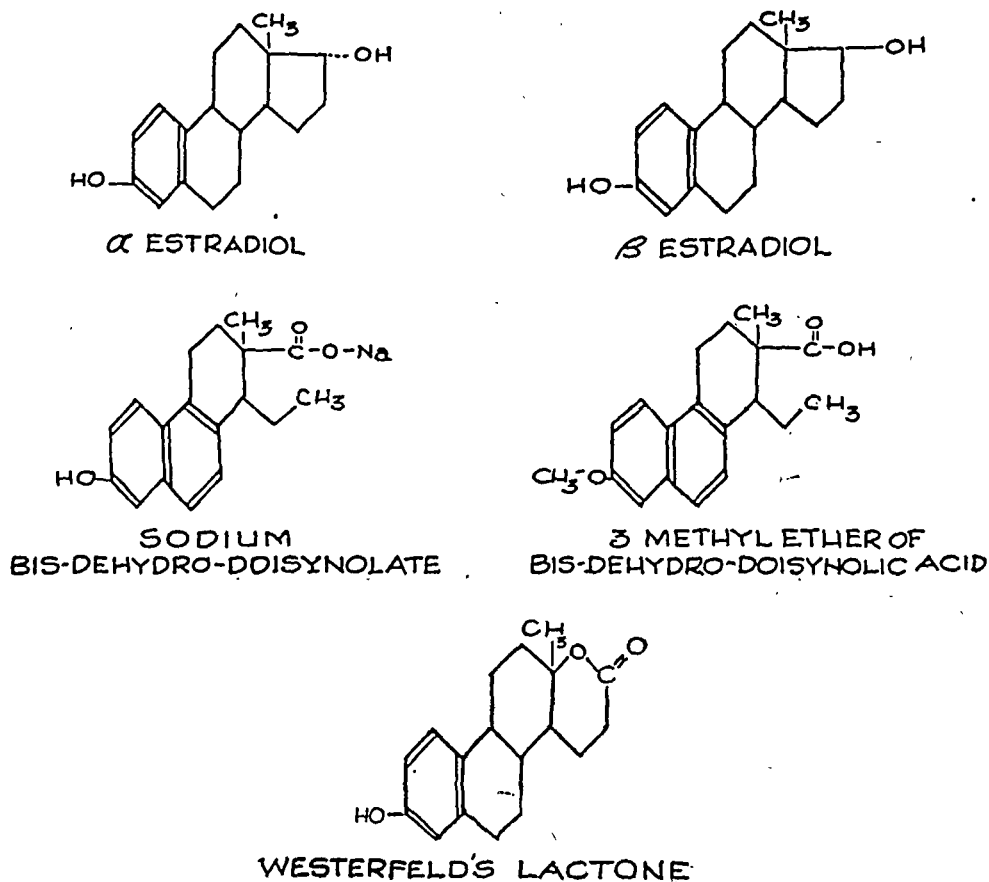
FIG. 1

situ. The 0.1 cc. contained 500 μg.; 0.1 cc. of the saturated solution of estrolactone acetate was also injected intrasplenically; this volume contained 1 mg. Vaginal smears were taken with a moistened cotton-tipped toothpick, 48, 54, 60 and 72 hours after the injection. The smears were air-dried and stained with hematoxylin and eosin. A smear which showed only cornified epithelial cells at one or more of these times was classified as representing a positive vaginal estrus response for that animal. At least 20 animals, but not more than 40, were employed at each of the levels being reported.

RESULTS

The results are presented in Table 1. The methyl ether of bis-dehydro-doisynolic acid and its sodium salt are considerably more active by subcutaneous injection than is α-estradiol. It is apparent

that Westerfield's lactone acetate is much less active by subcutaneous injection than is the sodium salt or the methyl ether of bis-dehydro-doisynolic acid. β-Estradiol has about the same activity by subcutaneous injection as Westerfeld's lactone acetate and the estrololactone acetate had far less activity by subcutaneous administration than any of the other compounds. In addition, Westerfield's lactone acetate is inactivated in the liver to a great extent since 500 μg. intrasplenically

TABLE I. THE ESTROGENIC ACTIVITY OF NATURAL ESTROGENS AND DEGRADA-
TION PRODUCTS THEREOF WHEN ADMINISTERED BY DIFFERENT ROUTES

	Subcutaneous injection		Intrasplenic injection		Ratio S.C.:I.S.
	Dose—	Estrus	Dose—	Estrus	
	μg.	%	μg.	%	
α-Estradiol	1.5	30	50	0	
	1.75	50	75	50	1:43
	3.0	85	150	90	
β-Estradiol	25	33	500*	20	
	40	50			
	75	72			
Westerfeld's lactone acetate. (From Drs. Pincus & Jacobson)	25	20			
	37.5	50			
	50	70			
Westerfeld's lactone acetate. (My preparation)	20	20	500*	10	
	30	50			
	37.5	67			
Estrololactone acetate	100	6	1000*	19	
	325	50			
	500	83			
3-methyl ether of bis-dehydro-doisynolic acid	0.20	10	0.10	0	
	0.35	55	0.20	50	1:0.57
	0.50	91	0.30	81	
Sodium-bis-dehydro-doisyno-late	0.5	10	0.26	30	
	0.75	45	0.375	50	1:0.5
	1.0	90	0.5	85	

* 0.1 cc. of a saturated solution.

with the spleen *in situ* elicited only a 10% estrus response. This is thirteen times the amount required for 50% estrus by subcutaneous injection, so that the ratio of subcutaneous to intrasplenic is greater than thirteen. Our preparation and that supplied by Dr. Pincus showed essentially the same activity by subcutaneous injection. β-Estradiol is also inactivated in the liver since thirteen times the 50% estrus dose by subcutaneous injection only produced a 20% estrus response. Therefore, the ratio for β-estradiol is also greater than thirteen.

Both sodium bis-dehydro-doisynolate and the 3-methyl-ether of bis-dehydro-doisynolic acid are more active when given intrasplenically than when given subcutaneously (Table 1). The subcutaneous-

intrasplenic ratios for the 50% estrus levels are 1:0.57 and 1:0.5 respectively.

DISCUSSION

- Westerfeld's lactone acetate seems to be metabolized in a manner similar to its parent compound, estrone, which was previously shown to be inactivated by the liver. In the studies on estrone the same technique was employed (Segaloff, 1943). This compound has aroused much interest since its preparation. Smith and Smith (1944) have demonstrated that despite its low estrogenic potency, it is more potent than estrone in its ability to increase the output of gonad stimulating hormone from the pituitary of the rat. The Smiths (1946) believe that Westerfeld's lactone, or some similar degradation product, plays a key role in the initiation of menstruation in man.

Estrololactone acetate also appears to be inactivated by the liver. Its low estrogenic activity, by a uterine weight assay method, as well as by vaginal smears, has been reported previously by Pincus (1947). He also reports that it is as potent as estrone in the inhibition of pituitary gonadotrophin, but unlike estrone it does not produce the release of pituitary corticotrophin.

β-Estradiol is also inactivated by the liver, but again its low potency precludes an exact estimation of the degree of inactivation.

The results with the methyl ether and sodium salt of bis-dehydro-doisynolic acid were surprising in view of the preceding discussion. It appears that the rat's liver is capable of activating or increasing the estrogenic potency of these compounds. We previously reported (1944) such *in vivo* activation of synthetic proestrogens.

It appears to be of great interest that rupture of the 5 membered ring in estrone can lead to compounds of such varying estrogenic potency which are handled by the liver in totally different ways.

SUMMARY

α-Estradiol and some estrogen degradation products were injected subcutaneously. It was observed that they could be arranged in the following order of decreasing estrogenic potency: 3-methyl ether of bis-dehydro-doisynolic acid > sodium bis-dehydro-doisynolate > α-estradiol > Westerfeld's lactone acetate > β-Estradiol > estrololactone acetate.

When α-estradiol was injected intrasplenically instead of subcutaneously, 43 times as much was required to produce vaginal estrus in one half of the animals. Intrasplenic injections of β-estradiol, Westerfeld's lactone acetate and estrololactone acetate with 12, 17 and 3 times the subcutaneous 50% estrus dose respectively failed to produce estrus in 50% of the animals. Intrasplenic injections of the 3-methyl ether of bis-dehydro-doisynolic acid and sodium bis-dehydro-doisynolate, on the other hand, required only one half as much to produce vaginal

estrus in 50% of the animals as when the estrogen was administered subcutaneously

These results are interpreted as indicating that the liver inactivates α and β-estradiol, Westerfeld's lactone acetate and estrololactone acetate but enhances the estrogenic potency (activates) the 3-methyl ether of bis-dehydro-doisynolic acid and sodium bis-dehydro-doisynolate.

ADDENDUM

Since this paper was submitted for publication a paper by Jacobson has appeared in which the preparation of estrololactone is discussed in detail. Such chemical data as are available are also discussed. (Jacobson, R. P. *J. Biol. Chem.* 171: 61. 1947.)

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VISCOSIMETRIC DETERMINATION OF THE HYALURONIDASE CONTENT OF SPERMATOZOA

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WITH THE observation that the dispersion of the cumulus cells surrounding the rat ovum was necessary to enable fertilization of the egg by sperm, and that this dispersion could be brought about by a heat labile substance produced by spermatozoa, Pincus & Enzmann (1935), the relationship of these substances to certain clinical aspects of sterility has received increasing attention.

Factors with similar diffusing properties have been obtained from numerous sources including culture filtrates, invasive bacteria, a large variety of organs and tissues, neoplasms included, snake venom and leeches. Chain and Duthie (1940) noted a marked mucolytic activity of these extracts, characterized by a rapid fall in the viscosity of synovial fluid and the liberation of reducing substances. Further investigation by others revealed that these spreading factors are closely associated, if not identical, with a group of enzymes that hydrolyze hyaluronic acid, and are known as hyaluronidases.

Rowlands (1944) demonstrated that with the addition of an enzyme from a semen filtrate, the number of spermatozoa required to accomplish artificial insemination in rabbits, was reduced one sixth. This work again suggested that the large number of spermatozoa was required for the fertilization of the mammalian ovum, in order to provide a concentration of enzyme sufficient to cause dispersion of the cumulus cells surrounding the ovum, so that one sperm could effectively fertilize the egg. The application of this concept to the study and treatment of human sterility (Eichenberger, 1946; Leonard and Kurzrok, 1945; Greenberg and Gargill, 1946; Swyer, 1946) has resulted in the need for a simple accurate method for the measurement of hyaluronidase content in fertile and infertile human semen.

The viscosimetric measurement of the degradation of hyaluronic acid by hyaluronidase in a system controlled with reference to pH and salt concentration, is probably the most reliable (McClean, 1941). For this reason, we undertook to adapt such a technique for

application in the study of the hyaluronidase content of semen. Our purpose was to maintain the level of accuracy and reduce to a minimum involved procedures and mathematical computations, so that the test could have clinical application. In addition to its accuracy, the technique below has the further advantage of requiring only small volumes of substrate and semen.

The hyaluronic acid solution is a 0.25% of purified material in distilled water. The hyaluronic acid is prepared from umbilical cords.[1] This solution has a viscosity of approximately four times that of water, when checked with the Ostwald viscosimeter. The solution is quite stable and will keep in the refrigerator for two to three months, with only a barely perceptible diminution in viscosity. The substrate is prepared by mixing four volumes of 0.25% hyaluronic acid solution with one volume of a Molar Sodium Citrate buffer pH 4.6. This buffer is prepared by mixing one volume of Molar Citric Acid with two volumes of Molar Sodium Hydroxide.

The viscosity of the substrate solution is determined with an Ostwald viscosimeter (approximately 5 ml. capacity). The substrate, composed of 2.5 cc. of 0.25% hyaluronic acid to which 0.625 cc. of Molar Sodium Citrate buffer pH 4.6 has been added, is mixed with 1 cc. of 0.1 Molar Sodium Chloride, and the entire mixture is poured into the viscosimeter bulb. All readings are done in a bath at 37.5° C. Several flow times are recorded and the average of these is the viscosity of the substrate solution. The value may be considered constant for the life of the hyaluronic acid solution (2–3 months).

Determination of the viscosity reducing effect of semen:

1. The specimen is collected in a chemically clean container which has been sterilized. Collected in this fashion, the specimen can be kept in a refrigerator for as long as two weeks with no apparent change in its viscosity reducing ability. Figure 1 demonstrates flow curves done on the same specimen two weeks apart. For the determination, the semen specimen is diluted with an equal volume of 0.1 Molar Sodium Chloride and centrifuged until the supernatent is clear of any particles.

2. One cc. of the supernatent fluid and the substrate solution described above, are warmed separately to 37.5° C. for ten minutes in the bath.

3. The preparation from the semen is added to the substrate, and at this instant stop watch #1 is started. (This watch runs throughout the determination and gives the total reaction time.) The solution is shaken vigorously.

4. Using a small glass funnel, the mixture is carefully poured into the lower bulb of the viscosimeter. The mixture is drawn up into the

tube and when the meniscus reaches the upper mark on the tube, stop watch #2 is started. The time it takes for the mixture to fall from the upper to the lower mark is referred to as the flow time, and is recorded by stop watch #2. At the end of each run, readings are taken on both watches and the flow time (reading on watch #2 for each run), is recorded against the total reaction time-reading on watch #1 which runs continually from the start to the finish of the determination.

TOTAL REACTION TIME
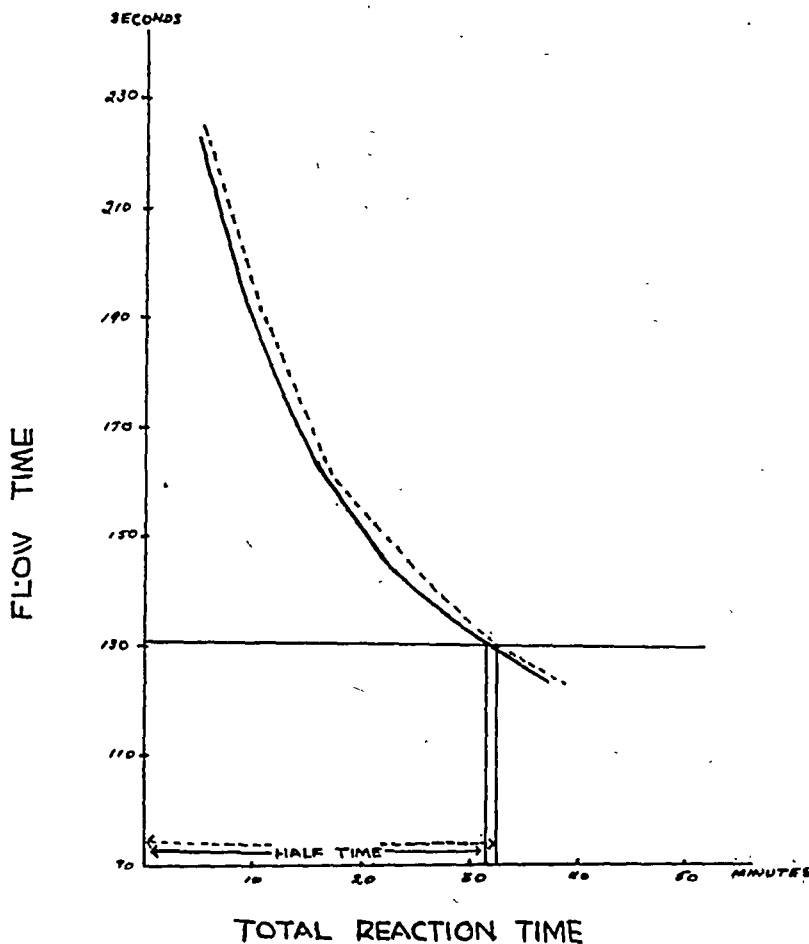
Fig. 1
Count 18,600,000 sperm/cc.
——— Feb. 1, 1947
-------- Same specimen Feb. 14, 1947.

The mixture is run at frequent intervals until the flow time of the enzyme system is less than one half that of the substrate. The end point is the total reaction time required to reduce the viscosity of the substrate to one half its original value.

Forty-four semen specimens were tested for enzymatic activity with this technique. The findings are graphically demonstrated in Fig. 2, in which the concentration of spermatozoa per cc. is plotted against the total reaction time required to reduce the viscosity of the

substrate to one half of its original value for each specimen. From this it is evident that but for three exceptions this time is inversely proportional to the concentration of spermatozoa per cc. Aspermic specimens failed to reduce the viscosity of the substrate. Figure 3 demon-
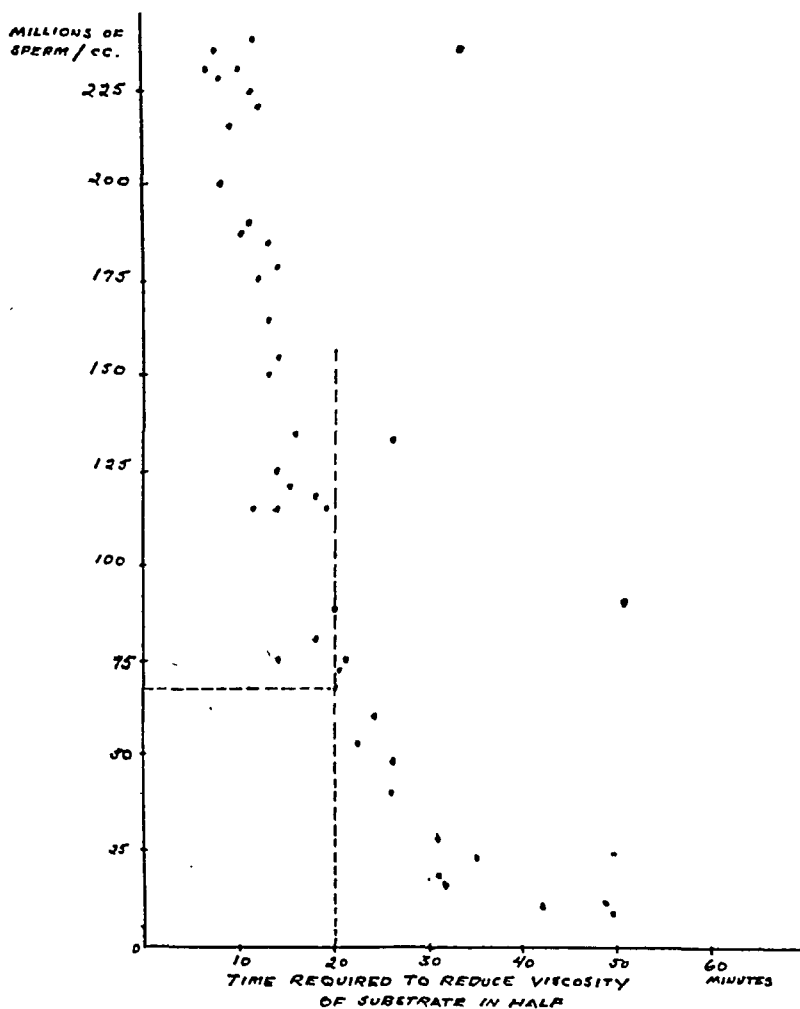
FIG. 2

strates characteristic flow curves for typical specimens. It is to be noted that all specimens, with the exception of the three noted above, containing more than 60,000,000 spermatozoa per cc. reduced the viscosity of the substrate to half value in 20 minutes or less.

Examination of the three specimens whose enzyme content could not be correlated to the sperm count, revealed some reason for the inconsistency in two of the specimens. One of these specimens, containing 215,000,000 spermatozoa was markedly viscus when received.

The viscosity six hours after it had been obtained by the patient was grossly much greater than normal. All other factors, volume, motility and morphology were within normal range. No evidence of prostatic infection was apparent in the specimen. This specimen, although with an apparent enzyme concentration characteristic of specimens con-
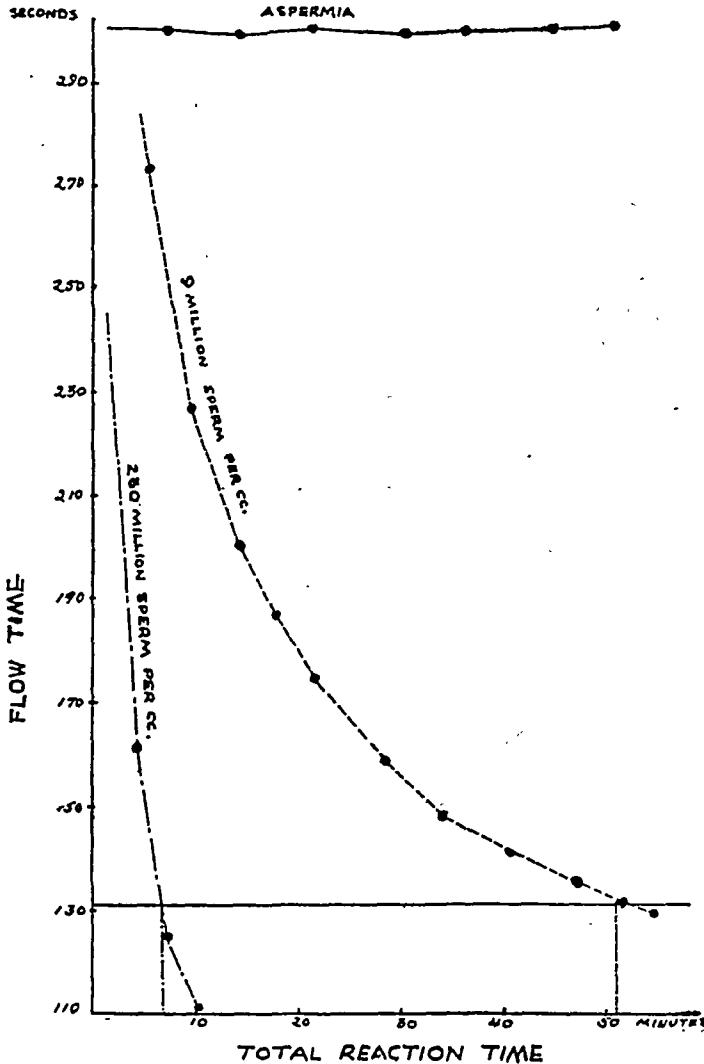
Fig. 3

taining less than 25,000,000 spermatozoa per cc. was from an individual who had manifest proof of his fertility. Repeat studies since then have yielded approximately the same results.

The second specimen contained 132,000,000 spermatozoa per cc. Volume, morphology and motility were within the normal range. Here too the viscosity was grossly much greater than normal. However in this instance there was definite evidence of a prostatic infection. Unfortunately we have been unable to maintain a follow-up on this patient.

The third specimen in this group contained 90,000,000 spermatozoa per cc. and was apparently normal in every other respect, volume, viscosity, motility and morphology. The concentration of the enzyme in this specimen is equivalent to that usually found in semen containing less than 15,000,000 spermatozoa per cc. Nothing is known concerning the fertility of this individual.

The concentration of hyaluronidase is apparently unrelated to morphology or motility. The apparent deficiency of the enzyme in very viscous specimens in spite of normal spermatozoa concentrations, indicates the need for more study in such instances. Fortunately one of these patients is available, and studies on the acid-phosphatase concentration of these specimens in conjunction with hyaluronidase estimations are being carried out at present. These findings will be reported at a future date.

Studies with aspermic specimens which failed to lower the viscosity of the substrate solution, reveal that these specimens have, however, the ability to diffuse in the skin when compared with a buffered control. These findings corroborate those of McClean (1941), that diffusion in the skin is caused by preparations that are too weak to be titrated viscosimetrically. Whether this diffusion is secondary to the presence of small amounts of hyaluronidase in the semen, or to other spreading factors, remains to be determined.

SUMMARY

A simple viscosimetric test for the determination of hyaluronidase in semen is described. The concentration of the enzyme as determined by this technique is proportional to the concentration of spermatozoa, with few exceptions. In two instances, abnormally viscous specimens failed to contain enzyme proportional to their sperm concentration. One semen specimen, apparently normal in all other respects, was also in this category. Aspermic specimens which could not be titrated viscosimetrically, demonstrated evidence of "spreading factor" when injected intracutaneously in the skin of rabbits.

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SOME FACTORS INFLUENCING THE LIBERATION OF HYALURONIDASE FROM TESTES HOMOGENATE AND SPERMATOZOA IN THE RAT[1]

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RECENT experiments concerning the effects of the enzyme haluronidase from sperm on mammalian ova have led to theoretical considerations of its role in reproduction (for discussion see Rowlands, 1944; Leonard, Perlman and Kurzrok, 1946). One of the problems has centered about the source of the enzyme obtained in the seminal plasma or extracts of testicular tissue, i.e., was it "secreted" by living sperm or germinal cells into the suspending medium, or was the enzyme liberation the reflection of a moribund cell population? The results of such studies would bear directly on any interpretation of semen assays as an index of fertility. It is evident from the experimental data to be presented that an increase in cell destruction is accompanied by an increase in the measurable hyaluronidase.

Methods

The methods of preparation of the testis homogenate and hyaluronidase determination were the same as previously described (Leonard et al., 1946). The enzyme unit is the turbidity reducing unit (TRU). It was possible to make a complete assay on a single testis and in many cases experiments were performed on one rat testis, the other serving as the control. Spermatozoa were taken from both the ductus deferens and the cauda epididymis of adult rats and were suspended in either Ringer's or 0.1 M acetate buffer (pH 6.0). Assays were made only on the cell-free supernatant fluid. Ejaculates were obtained, using a hypodermic syringe, from the uterus of a female rat following copulation.

It has been assumed that the source of the enzyme in the testis homogenate and sperm suspension was similar, i.e., the germ cells.

Hyaluronidase from ejaculates

The previous observation that the enzyme concentration in ejaculates collected from mated female rats was higher than that obtained from testes homogenates (Leonard, Perlman and Kurzrok, 1947) led us to investigate the variation which might occur in enzyme levels *in*

vitro at varying intervals following copulation. Within two minutes following copulation female rats were killed and the content of the uterus removed as rapidly as possible. The uterine fluid with sperm was diluted to 3–4 cc. with warm Ringer's solution. It was found that best results were obtained when the sperm count exceeded 10 million per cc. A sample was removed, centrifuged, and a hyaluronidase determination carried out on the supernatant fluid. The remainder of the suspension was stored at 37°C. for varying periods and then assayed. In four of five experiments a marked increase with time in

HYALURONIDASE ACTIVITY OF UTERINE CONTENTS FOLLOWING COPULATION

Exp.	Time until removal of cells	Sperm count (millions) per cc.	Turbidity reducing units per cc.	Calc. units per total uterine contents
A	20 min. 2.5 hrs. 13.0 hrs.	— 	50 66 90	25 33 45
B	20 min. 12 hrs.	— 	55 80	27.5 40
C	10 min. 1 hr.	56 56	24 80	6 20
D	9 min. 2 hrs.	56 56	<16 40	<4 10
E	7 min. 1.5 hrs.	18 18	17 20	8.5 10

the turbidity reducing units (TRU) of enzyme in the supernatant fluid was obsereved (Table 1). Microscopic examination of the sperm showed that a marked decrease in the motility was observed following incubation.

These results suggested that with decreased motility the increased amounts of enzyme were obtained from a dying cell population. Experiments were then devised to increase the enzyme liberated by subjecting the sperm or testes tissue to adverse treatment.

Hyaluronidase level from frozen testis and spermatozoa

If adult rat testes are homogenized soon after removal from the body and extracted in the cold (5°C.) for 30 minutes, the enzyme concentration obtained is 10 TRU per gram (average of more than 30 assays). Rat testes were frozen for 2 and 26 days, then homogenized and extracted. In four experiments, the yield of enzyme was 28, 24, 33, and 30 TRU per gram as compared to the 10 TRU per gram obtained with freshly removed tissue. No great difference was noted when varying the length of time the testes were frozen.

In a similar experiment, sperm suspensions in Ringer-phosphate

solution (pH 6.9) were prepared, using the sperm directly from the ductus deferens and cauda epididymis. One-half the sperm suspension was assayed immediately, the remaining half was alternately frozen and rapidly thawed three times within a period of several hours. The TRU per cc. of supernatant fluid in the control samples was 2, 3, 2, and that from the frozen sperm samples was 10, 10, 6, respectively.

Because previous results showed that incubation of fresh testis homogenate increased the hyaluronidase concentration of the extract (Leonard et al., 1947) *frozen* testes were homogenized and then incubated in order to ascertain whether or not the level of enzyme could be raised further. The incubation of frozen testes increased the TRU per gram of tissue in three experiments to 74, 69, 100 units in comparison with their respective controls (frozen but not incubated) which gave 28, 24, 26 units.

Effect of toluene and acid on testis homogenate

The two testes from each rat were weighed and homogenized separately. To one were added .75 cc. acetate buffer (pH 6.0) and .25 cc. of toluene, the other (control) received 1 cc. of acetate buffer only. After thorough mixing the homogenates were incubated for 3 hours (37°C.). In three experiments the TRU per gram of tissue in the controls were 53, 60, and 67 whereas the toluene-treated testes yielded 115, 110, and 104 units respectively. Furthermore, if the testes were frozen before incubating with toluene, an even higher level of enzyme was secured. The assay of a frozen, incubated testis was 90 TRU per gram; with toluene the other yielded 170 TRU per gram.

In one other experiment, the effect of lowering the pH of the testes homogenate on the subsequent yields of enzyme following incubation was observed. Four drops of 2 N acetic acid were added to one tube containing a single homogenized testis which lowered the pH below 4. The other testis from the same rat was homogenized and 4 drops of acetate buffer added. After 3 hours incubation at 37°C., the extracts were assayed. The TRU per gram obtained from the acid-treated homogenate in three experiments were 46, 56 and 42 units while the controls yielded 40, 51, and 39 units respectively. This indicated no significant increase in liberated enzyme as a result of the treatment with the acid.

<div align="center">DISCUSSION</div>

The first indication that increased amounts of hyaluronidase could be obtained from a dying sperm population was seen in the experiments in which the ejaculates were recovered from the uterus and kept in an incubator at 37°C. for varying periods. At first, this did not appear to be a particularly harsh treatment but the discovery of the decreased activity of sperm suggested that the liberation of the enzyme might well be enhanced under these conditions. Rat sperm do

not remain motile long when removed from the body and suspended in the usual physiological salt solutions. In this respect, rat sperm differ markedly from a number of other mammalian sperm (Chang, 1947).

It is clearly seen that the conditions under which these increased amounts of enzyme were obtained from the testes or sperm were unphysiological and, as such, any interpretation of the assay of hyaluronidase level in tissue or semen samples must be considered in the light of these results. In order to make comparable assays between samples, a rigidly controlled method of handling the samples previous to the assay seems indicated. Presumably, the more resistant the sperm are to adverse conditions the greater will be the likelihood that proportionately lower enzyme levels will be found in the surrounding medium. Whether this is true for all mammalian sperm remains to be determined.[3]

The enzyme-producing cells were given rather drastic treatment in order to determine the quantity of enzyme they were capable of yielding. Acetate buffer is not a good medium for maintaining viability but is the buffer of choice for hyaluronidase assay. Incubation at 37°C. for 3 hours even in what would be considered a favorable artificial environment is not conducive to longevity in rat sperm according to Chang (1947) and Yochem (1930). Freezing and thawing undoubtedly affected the cells in some way, resulting in an increase in the enzyme level of the surrounding medium. Toluene increased the enzyme concentration possibly by its action as a fat solvent in disrupting the integrity of the cell membranes, thereby freeing the enzyme into the surrounding medium (Meyer et al., 1941). In extracting hyaluronidase from skin he suggested also that autolysis of the tissue in the presence of toluene may favor the "conversion of an inactive to an active material." A combination of freezing, incubation and toluene treatment gave, in one experiment, the highest concentration of enzyme ever obtained from rat testes.

A different interpretation may have to be placed on our previous work in which augmentation of the enzyme secured from rat testes could be obtained by adding the seminal vesicle (Leonard et al., 1947). The facts remain unchanged as the experiment has been repeated many times with similar results. Unpublished data also show that augmentation can be demonstrated with suspensions of rat sperm and with the expressed fluid of the seminal vesicle. For the rat at least this also may be unphysiological because most of the seminal vesicle fluid is coagulated to form the vaginal plug and how much of it gains access to the uterus to mix with the sperm is not known.

[3] In a personal communication from Dr. John Mixner, it was pointed out that a similar situation was observed in bull semen samples. After recovery of the ejaculate, increases in enzyme concentration in the seminal plasma occurred with increasing loss of motility of the sperm.

droxide, rather than follow the procedure outlined in the U.S.P. of laking the blood in acid zinc sulfate.

Dilutions for injection were made with U.S.P. XIII diluting fluid adjusted to pH 2.8 to 3.0. The doses for subcutaneous administration were given in a volume of 1.0 cc. per rabbit while in the case of intravenous administration the doses were given in a volume of 2.0 cc. The dosage levels used were adjusted to comply with aperiodic variations in sensitivity and were arranged so that the high dose was twice the low dose, for example 0.90 and 1.80 International Units per rabbit. The effective dosage levels in the intravenous method were within the same range as those required for the subcutaneous method of administration. The doses were given in a random manner according to the 4×4 latin square design. In a few cases where accidents or death of a rabbit interfered with the completion of a square, another rabbit previously treated with insulin was substituted and these results were used in the completion of the square. Preliminary tests were conducted on all new groups of rabbits to accustom them to the bleeding and injection routine and to eliminate animals which appeared highly resistant or sensitive to insulin.

In the assays where subcutaneous administration was employed the rabbits were fasted for approximately 24 hours prior to injection, water was withheld during the test and the animals were used at weekly intervals.

In the case of intravenous administration food was withheld for approximately 16 hours prior to the test, water was available at all times and the rabbits were used daily for four consecutive days. In cases where an initial blood sample was used, this was taken from the marginal vein of one ear and the dose of insulin injected in the marginal vein of the other ear. Each rabbit in the group was injected in this manner at intervals of one or two minutes depending on the ease of bleeding. The final blood sample was taken from the marginal ear vein of each rabbit 50 minutes after the injection. Each day of the test the rabbits were fed after the final bleeding and permitted access to food for approximately 6 hours.

The results of the assays were subjected to analysis of variance as outlined by Bliss and Marks (1939) and the total variance was corrected for differences due to rabbits, days and doses.

RESULTS

The values shown in Table 1 were taken from assays performed using the U.S.P. Reference Standard against commercial samples of insulin prepared from amorphous insulin and zinc insulin crystals. The weighted mean value for the standard error (s) and slope (b) are shown in columns 4 and 5 respectively for each of the bleeding schedules indicated in column 2. In the group using the bleeding times 0 (initial) 1.5, 3 and 5 hours the assays were calculated first on the basis of percentage reduction (the mean decrease of the post injection sugar values as a percentage of the initial value) and secondly as mg. reduction (the mean decrease of the post injection sugar values) in which the initial value was excluded. Similarly the results from the same assays were recalculated to determine the effect of excluding the blood sugar value at 5 hours with and without the initial (0) blood

sugar value. The group of assays using the bleeding schedule of 0, 2 and 4 hours and the group using 0 and 50 minutes were treated in the manner described above. A final group of 24 assays where the initial blood sample was not taken are included for the purpose of comparison.

In order to obtain a measure of one treatment effect in terms of the other, the ratio of $(s/b)^2$ was calculated and is shown in column 6. Due to the relatively small number of assays on each of the treatment effects, the use of different groups of rabbits within and between the

TABLE 1. THE EFFECT OF THE BLEEDING SCHEDULE AND THE METHOD OF ADMINISTRATION ON THE SLOPE OF THE REGRESSION LINE AND THE STANDARD ERROR OF THE ASSAY

No. of assays	Bleeding schedule	Method of adm.	s	b	$(s/b)^2$
10	0,1.5,3 and 5 hrs.	subcut.	7.10	32.69	0.047
10	1.5,3 and 5 hrs.	subcut.	6.09	−28.49	0.046
10	0,1.5 and 3 hrs.	subcut.	7.75	36.91	0.044
10	1.5 and 3 hrs.	subcut.	6.36	−32.62	0.038
9	0,2 and 4 hrs	subcut.	9.21	39.34	0.055
9	2 and 4 hrs.	subcut.	7.49	−35.80	0.044
9	0 and 2 hrs.	subcut.	11.37	42.22	0.072
9	2 hrs.	subcut.	9.41	−37.98	0.061
5	0 and 50 min.	intraven.	11.27	52.53	0.038
5	50 min.	intraven.	9.11	−46.60	0.046
24	50 min.	intraven.	8.50	−40.63	0.044

different bleeding schedules and to the accumulation of the data over a period of three years it was not considered advisable to apply statistical tests for significance to the results, but to consider any difference as indicating trends.

It is seen that the ratio is lower in all cases, except the intravenous method of administration, when the initial value is excluded from the calculations. Similarly the ratio is lower when the bleeding at 5 hours is eliminated. These results indicate that the sugar value at 0 and 5 hours in the bleeding schedule 0, 1.5, 3 and 5 hours may be excluded without affecting the precision of the assay. The assays using the bleeding schedule at 0, 2, and 4 hours were designed to determine if this schedule was more efficient than the schedule using 0, 1.5, 3 and 5 hours. Since the ratio is higher for the former no advantage is indicated, except in reducing the number of bleedings to 3 instead of 4 and shortening the duration of the test one hour. The highest value for the ratio was obtained with the bleeding schedule at 0 and 2 hours. The ratio for $(s/b)^2$ using the intravenous administration and one bleeding at 50 minutes is well within the range of the values found for the subcutaneous administration. Since only one quarter of the blood samples are required in the intravenous method the economy of material and effort is quite apparent and there is considerable saving of time to complete the latin square cross-over design. In this instance

it requires four days as compared to four weeks for the subcutaneous method. The latter method does not permit a feeding period on a test day while in the intravenous method the test can be completed quite conveniently in 2 hours permitting the rabbits to feed for 5 to 6 hours and then withdrawing the food over-night.

The rabbits on each of the treatment effects were weighed at weekly intervals. The results plotted in Figure 1 show that the rabbits
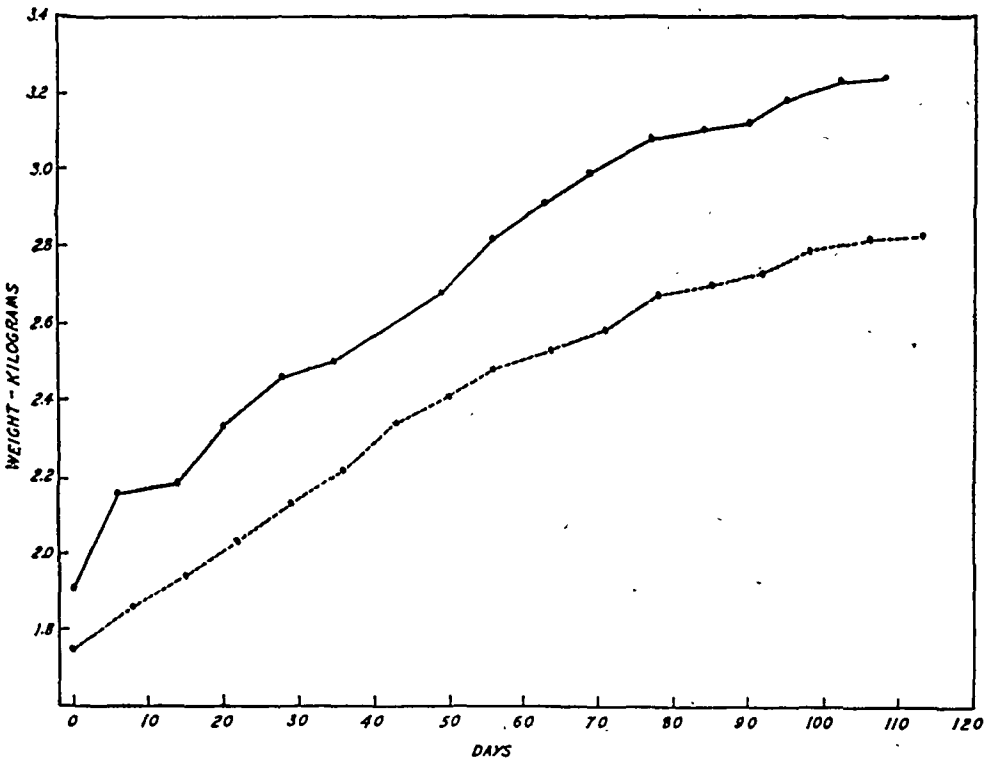
FIG. 1. The effect of the administration of insulin on the body weight of rabbits. Solid line = intravenous injection; broken line = subcutaneous injection. Each point represents the mean body weight of 14 rabbits.

on the intravenous administration gained in weight at approximately the same rate as the rabbits on the sucutaneous method of administration.

The daily bleeding and intravenous injection resulted in no more injury to the ears of the rabbits than was experienced with the subcutaneous method of administration using four bleedings. There was no evidence of the development of tolerance to the intravenous administration of insulin.

Six different samples of insulin supplied by different manufacturers were assayed first by the subcutaneous method using the bleeding schedule at 0, 1.5, 3 and 5 hours and secondly, by the intravenous method using one bleeding at 50 minutes. The results in Table 2 show

TABLE 2. A COMPARISON OF POTENCY OF COMMERCIAL SAMPLES OF INSULIN
ASSAYED BY SUBCUTANEOUS ADMINISTRATION AND BLEEDINGS AT
0,1.5,3 AND 5 HOURS WITH INTRAVENOUS ADMINISTRATION
AND BLEEDING AT 50 MINUTES

Sample No.	Labelled potency in I.U.	Subcutaneous				Intravenous			
		b	s	$(s/b)^2$	Potency found in I.U.*	$-b$	s	$(s/b)^2$	Potency found in I.U.*
1	20	44.12	7.69	.030	*21.8* (17.9 to 26.6)	40.53	6.46	.025	*20.4* (17.0 to 24.4)
2	20	41.76	6.29	.023	*20.5* (17.1 to 24.1)	49.14	6.81	.019	*21.1* (18.0 to 24.7)
3	20	33.29	7.30	.048	*23.2* (18.0 to 29.9)	33.22	8.05	.059	*20.4* (15.6 to 26.6)
4	20	24.65	7.04	.081	*21.1* (15.3 to 29.1)	40.26	7.57	.035	*21.1* (17.0 to 26.1)
5	40	32.22	5.54	.029	*36.6* (29.9 to 44.8)	46.37	7.57	.026	*31.5* (25.9 to 38.2)
6	40	22.03	6.49	.086	*38.4* (27.5 to 53.5)	35.35	7.10	.040	*42.4* (33.7 to 53.2)

* Mean potency in italic type, range expressed as 1.96×Sm.

there is good agreement in the estimation of potency and in the precision of assay by the two methods. The value (b) for the slope of the regression line in the case of the intravenous method, where mg. reduction is used as the response, is negative on account of the inverse relationship between dose and response.

DISCUSSION

The majority of the reports on the assay of insulin have been concerned either with the effect of variations in the design or with methods of interpreting the assay results without much consideration of the bleeding schedule. Recently DeJongh et al. (1947) have reported their results on the correction factor for the initial blood sugar. They have shown that equally satisfactory results can be obtained by correcting the initial sugar determination and basing the results on the absolute value of the decreased blood sugar, or by expressing the results in terms of per cent of the initial value. In both cases they found a highly significant correlation between the initial blood sugar and the reduction to be obtained after subcutaneous administration and a bleeding schedule at .75, 1.5, 2.25 and 3 hours after injection. The results presented in Table 1 indicate that the uncorrected values for the initial blood sugar do not contribute to the precision of the assay, and in the interests of efficiency may be omitted. Admittedly it would be advisable to conduct control blood sugars on new groups of rabbits in order to rule out any that show abnormalities in this respect. Since the labor and expense involved in the assay of insulin are relatively greater than in most assay procedures, any reduction in these factors appear to be justified. In this connection Bliss and Bartels (1946) in a preliminary report using the discriminate function test claim that the different time intervals in the bleeding schedule of the insulin assay are

markedly unequal in the information they provide on the potency of the product. Although the results indicate that the 5 hour sugar value does not contribute to the precision of the assay, however it is of value in providing qualitative information on the reaction of the rabbit to insulin. In common with most biological assays this method leaves much to be desired with respect to the use of the product clinically, for the rates of absorption and utilization of insulin in the diabetic individual is undoubtedly different from these rates in the normal rabbit. Hence, the justification for a method of assay that ensures uniformity of a product. Thus far no attempts have been made to correlate the rabbit assay method with a method using diabetic patients.

The method of assay reported by Young (1945) and Young and Romans (1947) using intravenous administration of the doses and one bleeding 50 minutes after injection gives results (Table 2) in good agreement with those obtained by the subcutaneous method of administration. The economy of time and effort of the former is shown in Table 1. The method was found very practical and no untoward effects were observed in the rabbits. Normal weight gain was maintained (Figure 1) and it has been our experience that the incidence of convulsions in assays is considerably lower than with subcutaneous method of administration. Young (1945) has studied the dosage response relationships and has shown that the method as outlined above complies with accepted principles of a satisfactory assay. The two dosage levels of standard and sample employed in this work yield a limited amount of data on this point, but in all the assays reported here a significant slope was found and in no case was there evidence of non-parallelism between standard and sample. Young (1945) made a preliminary study of bleeding times at 45, 60 and 110 minutes after injection and the results indicate that these bleeding times do not offer any advantage over the bleeding schedule at 50 minutes. The latter is a very practical time in the conduct of the assay, but undoubtedly this point deserves further study.

SUMMARY

In the assay of insulin using rabbits the results indicate that the efficiency of the method can be considerably improved without loss of precision by omitting the initial and final blood sugar determinations in the conventional bleeding schedule. Considerable economy of time and effort without loss of precision can be accomplished in the assay of insulin by intravenous administration of the doses and using one bleeding 50 minutes after injection. The potency of commercial insulin preparations when assayed by the intravenous administration of the doses showed excellent agreement with the potency determined by the subcutaneous administration of the doses and a bleeding schedule at 0, 1.5, 3 and 5 hours.

ACKNOWLEDGMENTS

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EFFECT OF RECIPROCAL STEROID TREATMENT ON THE ELECTROPHORETIC PATTERNS OF FOWL SERA

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MARKED differences in the electrophoretic patterns of cock and hen sera were reported in 1945 (Moore). It is the purpose of this communication to trace the development of these differences beginning with the young chick and to report the changes produced by injecting the adult fowls with contra-sex hormones.

METHODS

Blood was taken from the heart by inserting a syringe needle in front of the furculum into the thorax. By this method 5 to 10 cc. of blood could be taken without apparent injury to the bird. The sera were analyzed in a buffer 0.02 M with respect to sodium phosphate and 0.15 M with respect to NaCl at pH 7.4 (Tiselius and Kabat, 1939). Each serum sample was diluted with two parts of the buffer and dialyzed in a bag made from viscose sausage casing (average pore diameter 2.5 mμ) against one liter of the buffer for 24 hours, whereupon the volume was re-measured in order to correct for any change during dialysis. The conductivity and pH of both the diluted sera and dialysate were routinely taken. The conductivities were used in calculating the mobilities of the various electrophoretic components.

The analyses were carried out in a Tiselius apparatus (Tiselius, 1937) having a single-sectioned tall cell of 2 cc. capacity. The center section is 50 mm. tall, 15 mm. deep (along the optic path) and each channel is 2 mm. wide. The channels are 10 mm. apart (center to center). A current of 18.7 milliamperes flowed through the cell and produced a field strength of 6.5 volts/cm. All patterns were obtained by the scanning method of Longsworth (1939) on panchromatic plates (Kodak M), the light source being a 200 watt tungsten projection lamp. If photographed with uniform exposure, the area of a pattern component is proportional to the product of the specific refraction and the concentration of the corresponding serum component.

EXPERIMENTAL

The chickens used in these experiments were Rhode Island Reds. Distinct sex differences in the serum patterns were not apparent until the fourth or fifth month of life, hence the hormone experiments were not begun until the 175th day of life, which was after the hens had started laying. At this time 2 cocks were injected intramuscularly

with 0.1 mg. α-estradiol dipropionate (Di-ovocylin, Ciba) in sesame oil and 3 hens were injected with 0.2 mg. testosterone propionate (Perandren, Schering) daily for 14 days. The sera immediately before and after this treatment were compared.

After several weeks the experiments were continued with larger doses of hormones and young adult capons were substituted for the cocks. Two of the hens received 1 mg. testosterone propionate daily for 18 days, while the third received an equivalent volume of sesame oil as a control. The three capons, after being bled for control sera, received 1 mg. Di-ovocylin per day for 18 days.

Some samples of sera were extracted with ether by the method of McFarlane (1942). Two volumes of serum were mixed thoroughly with 1 volume of chilled ether and quickly frozen in a dry ice-alcohol bath. The sample was then allowed to thaw and separate in the ice box for 8 hours or longer, whereupon the serum was carefully removed from underneath the ether layer with a syringe and blunt needle. This process was repeated two additional times. Residual ether was removed by a short period of vacuum desiccation. McFarlane reports that this process removed only part of the total lipids but apparently does not denature the proteins. A comparison of nitrogen (determined by micro Kjeldahl method) with pattern areas before and after ether extraction indicated that the ratio of pattern areas to nitrogen was reduced by 15 to 25 per cent by the extraction process. This result shows that a large quantity of material having a high refractive index to nitrogen ratio was removed by the ether.

OBSERVATIONS

The data of Tables 1 and 2 are summarized in the patterns of Figure 1. It is apparent that pronounced sex differences in the sera do not appear in the chicken until maturity and become more pronounced with age. The hens in these experiments began laying between the 155th and 170th day. The administration of 0.1 mg. androgen daily for 14 days in the hen did not produce significant changes in the serum except that the component of highest mobility was reduced. This component was never present in cock serum and disappeared upon extraction with ether in hens. Administration of 0.2 mg. estrogen for the same period in the 175 day old cocks did, however, produce appreciable changes in the serum. Component 2 virtually disappeared, the fastest moving component began to appear and the lagging end of the pattern took of the spikey character of a hen serum pattern (see Figure 1, low hormone injected). Encouraged by these results the daily injections were later increased to 1 mg. and adult capons were substituted for the cocks. At the beginning of this latter experiment the hens were 260 days old and had resumed laying some days after cessation of the 0.1 mg. androgen injection. During this rest period the lagging components of the serum pattern had greatly increased in size assuming the

TABLE 1. ELECTROPHORETIC DATA ON NORMAL HEN AND COCK
SERA AT VARIOUS AGES

Identification	Pattern Areas Arbitrary units							Mobilities*					
	f	1	2	3	4	5	Total	f	1	2	3	4	5
Pullets and Cockerels at 42 Days and 84 Days of Age													
42da ♀		195	50	40	35	25	345		5.2	4.4	3.4	2.1	1.3
42da ♂		180	70	35	50	40	375		5.1	4.1	3.2	2.4	1.2
84da ♀		140	95	25	95		355		5.2	4.3	3.4	2.5	
84da ♂		185	80	25	80		370		5.3	4.3	3.4	2.6	
100 Day Pullets													
#2		235	70	20		190	515		5.0	4.2	3.1		1.6
#4		250	80	35	105	45	515		5.0	4.2	3.1	1.8	1.1
#6		220	70	15	80	30	415		5.0	4.2	2.9	1.8	1.2
100 Day Cockerels													
#1		215	95	30	150		490		5.0	4.2	3.1	1.8	
#3		180	100	20	120		420		5.0	4.2	3.1	1.8	
#5	5	205	100	40	70	30	450	6.0	5.0	4.2	2.9	1.6	1.0
135 Day Pullets													
#2		210	60	30	125	75	500		4.7	3.8	2.9	1.6	1.0
#4		180	70	25	50	40	365		4.8	3.9	3.0	2.4	1.2
#6		215	95	45	140		495		5.0	4.2	3.3	2.3	
135 Day Cockerels													
#1		230	120	50	100	35	535		5.2	4.2	3.3	1.8	1.1
#3		160	100	30	65	25	380		5.2	4.2	3.3	2.0	1.5
#5		235	110	45	80	35	505		4.9	3.9	3.0	2.4	1.2
175 Day Hens													
#2	90	320	30	40	270		750	6.3	5.1	4.2	3.1	1.8	
#4	45	335	25	65	100	85	655	6.5	5.2	4.2	3.1	1.9	0.5
#6	45	340	15	25	170		595	6.1	5.1	4.2	3.1	1.8	
175 Day Cocks													
#3		180	95	20	90	20	405		5.2	4.2	3.2	2.2	1.8
#5		170	75	30	120		395		5.3	4.4	3.4	1.9	
175 Day Hens—Ether Extracted													
#2		270		30	105	75	480		4.8		3.0	1.7	1.2
#6		290		30	115	20	455		4.8		3.1	1.9	1.0
175 Day Cocks—Ether Extracted													
#3		205		60	40	10	315		4.9		3.1	1.8	1.1
#5		125		70	40	20	255		4.9		3.0	1.8	1.1

* Mobilities derived from descending patterns are expressed in units of 10^{-5} cm.^{2}/volts-sec. All components are anodic.

proportions previously reported (Moore, 1945) for mature laying
hens (see Figure 1, control). After 18 days of the high androgen ad-
ministration, the hen serum pattern was almost identical to the typical
pattern of cock serum. The fastest component had receded, a normal
cock serum component 2 had appeared and the lagging portion of the
pattern had diminished and taken on the contour of a cock serum pat-
tern. Similarly, the capon serum which was already different from
normal cock serum, was almost transformed into a typical hen serum
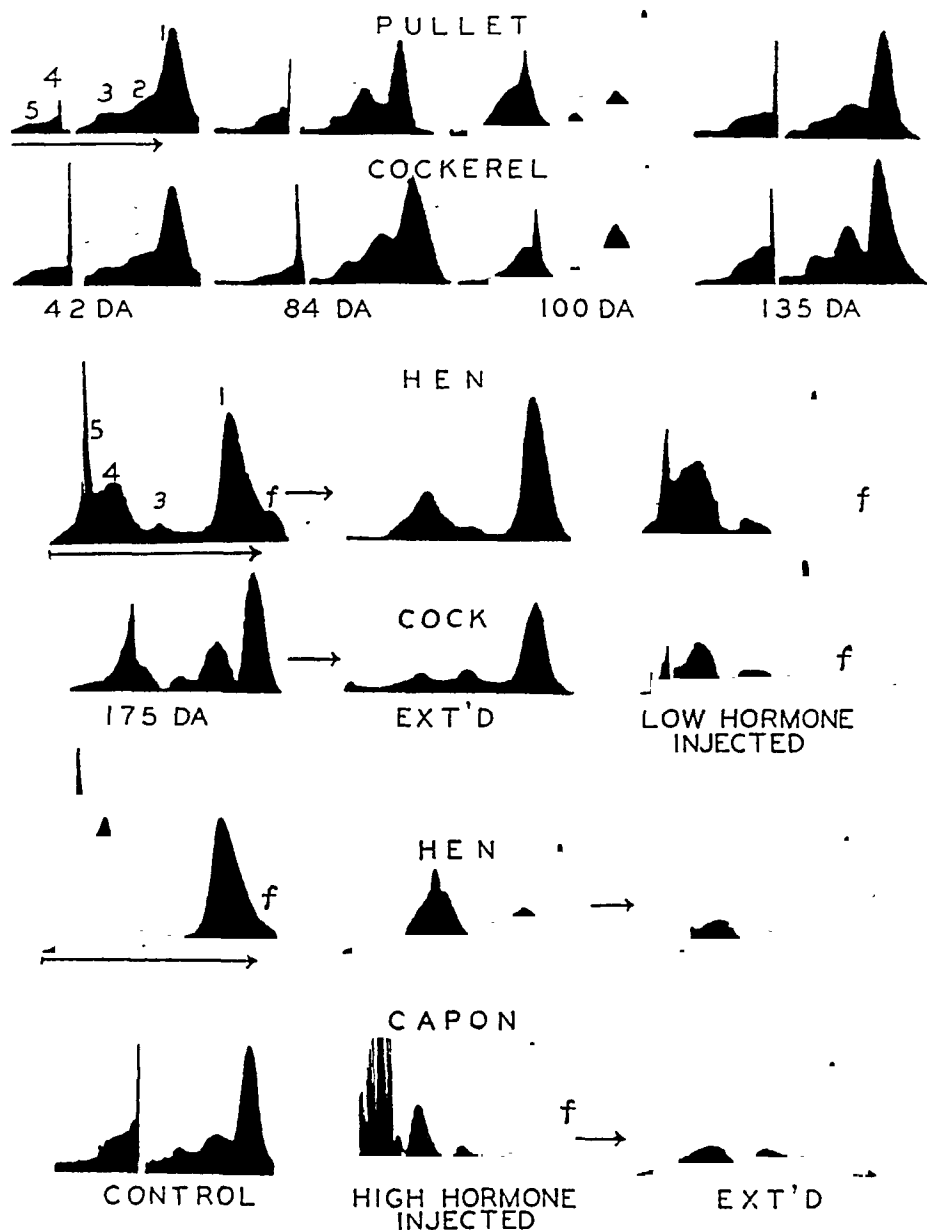
FIG. 1. Electrophoretic patterns of female and male chicken sera showing similarities during the development period (up to 135 days); difference after maturity (175 days), same sera ether extracted (ext'd), changes produced by 0.2 mg. testosterone propionate and 0.1 mg. α-eatradiol dipropionate daily for 14 days in the hen and cock respectively (low hormone); control hen and capon at 260 day (control); after 1 mg. per day in both hen and capon for 18 days (high hormone), and same sera ether extracted (ext'd).

(cf. high hormone injected capon with control hen of Figure 1 and also with laying hen of Figure 2).

From the patterns of ether-extracted sera it is apparent that the substance responsible for sex difference is largely removed by the ether extraction process. The fastest component in the 175 day normal hen serum and component 2 in the normal cock serum disappeared, leaving the leading ends of the pattern identical in respect to number of components. Component 3 was larger, however, in the extracted cock
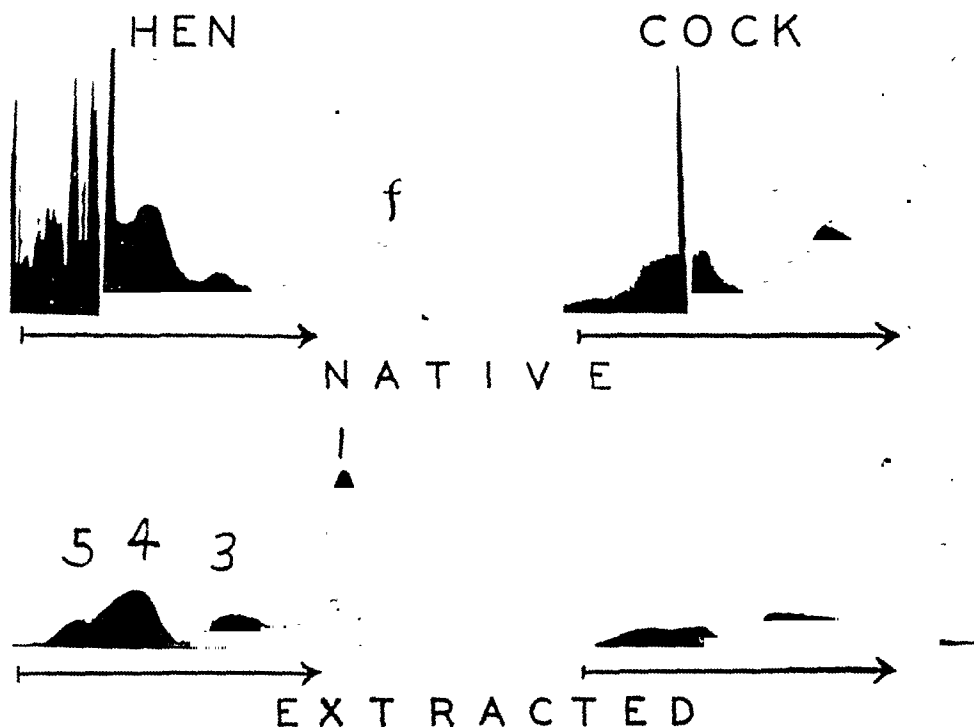
FIG. 2. Laying hen and normal cock serum patterns before (native) and after (extracted) ether extraction.

serum than in the extracted hen serum, but components 4 and 5 were much larger in the hen serum, component 5 being almost absent in the extracted cock serum. After hormone treatment ether extraction rendered the hen and capon sera almost indistinguishable (see Figure 1, high hormone, extracted).

In order to ascertain that the reduction in pattern area was not accompanied by a similar reduction in protein nitrogen, a comparison of electrophoretic pattern area with nitrogen content was made before and after ether extraction. The data are presented in Table 3. Kjeldahl measurements were made on the diluted and dialyzed material that went into the electrophoresis cell. The ratio of pattern area to nitrogen content was reduced more in laying hen serum than in normal cock serum.

TABLE 2. ELECTROPHORETIC DATA ON HEN AND COCK SERUM AFTER STEROID TREATMENT

| Identification | Pattern areas Arbitrary units | | | | | | | Mobilities* | | | | | |
---	f	1	2	3	4	5	Total	f	1	2	3	4	5
190 Day Hens After 0.2 mg. Testosterone Propionate Daily for 14 Days													
#2	50	285	25	50	180	130	720	5.6	4.6	3.4	2.9	1.6	1.1
#4	20	250	30	30	55	95	480	5.5	4.8	3.6	3.0	1.8	1.5
#6		260	55	55	170	35	585		5.1	4.0	3.3	1.8	1.4
190 Day Cocks After 0.1 mg. α-estradiol Dipropionate Daily for 14 Days													
#3		155	50	30	100	20	355		5.0	4.1	3.2	1.9	1.4
#5		160	90	30	65	20	365		5.0	3.9	3.3	1.9	1.4
Hens After 1.0 mg. Testosterone Propionate Daily for 18 Days													
#2C†	60	280		50	270	245	905	5.9	5.0		3.2	1.9	1.1
#4		200	75	55	210		540		5.0	3.9	3.2	1.9	
#6		205	50	40	180		475		4.9	4.0	3.2	1.9	
Capons—Before and After 1.0 mg. α-estradiol-Dipropionate Daily for 18 Days													
#A Before		220	70	20	45	80	435		5.2	4.1	3.3	2.0	1.3
#A After	10	250	40	35	100	90	525	6.0	5.0	3.9	3.0	1.8	1.2
#B Before		210	70	25	50	50	405		5.2	4.2	3.2	2.1	1.3
#B After	20	220	60	50	125	150	625	6.1	5.4	3.9	3.4	2.1	1.4
#C Before		190	65	30	80	50	415		5.1	4.2	3.2	2.6	1.4
#C After	30	280		75	130	240	755	6.1	5.1		3.2	2.1	1.5
Ether Extracted After 1 mg. Hormone Daily for 18 Days													
♀ #6-4		215		60	65	20	360		5.2		3.4	1.7	1.1
Capon #A		220		60	100		380		5.0		3.2	1.8	

* Mobilities derived from descending patterns are expressed in units of 10⁻⁵ cm.²/volts-sec. All components are anodic.

† Control injected with sesame oil without hormone.

TABLE 3. COMPARISON OF SERUM ELECTROPHORETIC PATTERN AREAS WITH KJELDAHL NITROGEN BEFORE AND AFTER ETHER EXTRACTION

| Serum | Pattern area-arbitrary units | | | | | | | Total nitrogen Kjeldahl mg/cc | Pattern area to nitrogen ratio | Change in ratio % |
---	f	1	2	3	4	5	Total			
Normal Laying Hen										
Native	75	270		50	130	220	745	2.45	305	
Extracted		240		45	60	35	375	1.59	235	−23
Normal Adult Cock										
Native		250	S5	40	70	50	495	1.87	265	
Extracted		255		65	25	5	350	1.58	220	−17

DISCUSSION

Sex differences in the electrophoretic pattern of fowl sera have been confirmed. Deutsch and Goodloe (1945) state that "a slight difference between male and female plasmas was apparent." The differences previously reported from this laboratory (Moore, 1945) and those reported now show marked sex differences. The failure of Deutsch and Goodloe to observe significant sex differences may have been due to their employment of a barbiturate-sodium citrate buffer at pH 8.6 or they may not have obtained plasma from fully mature birds.

The comparison made here of chicken serum before and after ether extraction illustrates the enormous quantity of lipoid substance it contains, and also indicates that the sex differences observed in the electrophoretic patterns are largely removed upon ether extraction. Zeldis, Alling, McCoord and Kulka (1945) have shown that the removal of lipids by an alcohol-ether extraction method described by Blix (1941) reduces the electrophoretic pattern area to protein-nitrogen ratio in both human and dog plasma. In hyperlypenic dogs the reduction was principally in the α-globulin, although the pattern area of all the components was usually reduced. β-globulin, however, was the principal loser in the several pathological human plasmas examined. McFarlane (1942), in his studies on normal human serum, reported that by the method of lipid extraction used in this work only β-globulin is reduced. In chicken serum, however, this ether extraction process reduces the area represented by several electrophoretic components and causes some of them to disappear completely (e.g., the f-component in hen and the α-component in cock sera). Not only lipids but also some protein is removed as is evidenced by the decrease in nitrogen after extraction (see Table 3). This probably indicates that the sera contain firmly bound lipoprotein complexes which are predominantly lipid and therefore extractable by the method used.

The rapid increase in pattern area (see Table 1, 135–175 days) as the hen begins to lay is consistent with the increase in body fat at this period, reported by Halnan and Cruickshank (1933). In view of the researches of Zondek and Max (1939), Lorenz (1938 and 1943) and Flock and Ballman (1942), which demonstrated artificially-induced rapid fattening of male chickens by administration of estrogens, it is not surprising that serum from normal hens or estrogen-injected cocks or capons contained a large amount of lipid. However, the pattern for normal adult cock serum is also appreciably reduced upon ether extraction, and component 2 completely disappears, indicating that lipids or lipoproteins may be present in components not presenting a sharp or spiked pattern.

SUMMARY

Sex differences in the electrophoretic pattern of chicken serum appeared at the beginning of sexual maturity. Hen and cock serum

patterns were reversed by contra-sex hormones. Ether extraction indicated a high lipoprotein content of both cock and hen sera which was mainly responsible for the electrophoretically-observed differences.

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ANATOMICAL COMPARISON BETWEEN THE ADRENAL GLANDS OF WILD NORWAY, WILD ALEXANDRINE AND DOMESTIC NORWAY RATS[1]

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OUR KNOWLEDGE of the effects produced by domestication on the endocrine system of animals is very limited, owing in large part to the fact that domesticated and wild forms of the same animal are rarely available simultaneously. The common Norway rat, however, constitutes a definite exception to this statement, since it has many representatives of both domestic and wild forms alive at the present time. Domestic Norways have been reared in laboratories throughout the world for the past 60 years or more, while the wild Norways still live in alleys, yards, cellars and houses and also have a world wide distribution. The domestic rats are tame, tractable, do not attempt to escape, and reproduce well in captivity; the wild rats usually live in burrows in the ground and are fierce, savage, suspicious, and make every effort to resist captivity. Except under particularly favorable conditions, they have not been successfully reared in captivity.

The common Norway rat thus provides excellent material for studying the effects that are produced by domestication. From the great variety of experiments carried out over such an extended period of time we probably know more about the domestic Norway rat than about any other animal. However we know very little about its wild relatives even though they are available for study in large numbers almost at our doorstep.

Until recently the lack of a simple method of catching wild Norway rats alive and in large numbers prevented the carrying out of such a study. During the war, when large numbers of wild rats were needed for screening of rodenticides, we put into use an inexpensive, easily operated trap that made it possible to catch wild Norway rats with considerable ease and certainty (Richter and Emlen, 1945).

Several comparative studies on the anatomy, pharmacology and physiology of these two strains have already revealed marked differences between the domestic and wild forms (Dieke and Richter, 1945; Fish and Richter, 1946; Griffiths, 1944 and 1947). The present study concerns the effects produced on the adrenal glands:

H. H. Donaldson in 1924 reported that recently trapped wild rats had heavier adrenals than did his domestic rats. In 1928 J. C. Donaldson reported that an increased amount of cortical tissue accounted for this difference.

We have made a more extensive study of the adrenals in these two strains: (1) on growth of adrenals with relation to body weight; (2) on relative weights of cortex and medulla of wild and domestic forms and the effects produced on these relative weights in wild rats by extended periods in the laboratory on a regular laboratory diet; (3) on changes that result from crosses between wild and domestic rats.

For purposes of further orientation in adrenal size of wild rats, studies were made also on the wild Alexandrine rat. This animal, next to the wild Norway, is the most common rat throughout the world. Compared to the Norway it has a longer tail, larger ears, and a sleeker and more streamlined appearance. It inhabits ships, seaport towns, and cities, and tends to live and make its nests in ceilings and rafters rather than in burrows. This rat has not been domesticated. It was not possible therefore to obtain any data relating to the domestic form of this animal.

MATERIAL AND METHODS

Our colony of domestic Norway rats originally came from the Wistar Institute. Since then, except for a few pigmented animals added in 1927, no rats from other strains have been introduced. The wild Norways and most of the Alexandrines, other than those designated as being born in the laboratory, were trapped in the city of Baltimore and examined as soon as they were received. A few of the Alexandrines came from Florida.[3]

To detect any seasonal effects, we made a separate comparison of the adrenal weights of wild Norways killed in the summer and in the winter months.

The adrenal glands were dissected out immediately after death and weighed on a torsion balance to the nearest tenth of a milligram.

A total of 107 domestic Norways, 147 wild Norways and 69 wild Alexandrines were used to obtain the adrenal growth curves. In each instance these rats were evenly distributed over the entire body weight range (none below 25–50 grams).

The relative weights of cortex and medulla could not be determined directly because an accurate separation of the adrenal into its constituent parts for weighing is impossible. An indirect method was therefore resorted to, namely the comparison of the wieght of paper cut to conform with outlines of the cortex and medulla as projected from histological sections of the glands.

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[3] J. Spencer of the Fish and Wildlife Service at Gainesville, Florida, kindly trapped and sent these rats to us.

After sectioning, at 20μ, and staining with hematoxylin and eosin, every fifth section was projected by means of an Edinger projection machine onto No. 3 Green Spring bond paper and the outlines of both the cortex and medulla were traced at a magnification of 45× (Jackson, 1919, modified). A total of more than 60 projections was thus obtained for the smallest set of adrenals and many more for those of the larger rats. The tracings were cut out and all weighed on the same day to avoid errors which might arise from variations in atmospheric conditions. The medullary parts of each tracing were then cut free from the cortex and weighed again. The relative percentages of the medulla and cortex were thus determined. The actual weight of each part was then calculated from the known weight of the whole gland.[4]

For the determination of the cortico-medulla relationship we used 2 males and 2 females from each of the following 5 groups: domestic Norways, wild Norways killed soon after capture, wild Norways born in the laboratory and kept under laboratory conditions for about a year, wild Alexandrines killed immediately after being received from Florida (2 males and 1 female), and wild Alexandrines born in the laboratory and kept for about a year. The exact age of the wild freshly trapped rats could not be determined, but by comparing the weights of the animals caught in the field with those of known age brought up in the laboratory, we have estimated that the wild Norways were one year old or a little less. We took into consideration the fact that wild rats living under laboratory conditions are generally heavier than those living in the wild state and that a domestic rat tends to be fatter than a wild one brought up in the laboratory. The domestic Norway males used weighed in the neighborhood of 300 grams; the wild Norways from the laboratory 250 grams; and those from the street 225 grams. The Alexandrines born in the laboratory averaged 225 grams and those from the street 150 grams. The females weighed slightly less than the corresponding males; none of them was either pregnant or lactating. Only rats in apparent good health were utilized, as it is well known that infection will greatly increase the size of the adrenal cortex (Grollman, 1936).

<div align="center">RESULTS</div>

The photographs of adrenal glands in Figure 1 show the effect that domestication has on the size of this gland. In typical female animals weighing about 250 grams the left adrenal of the domestic Norway weighed 32.4 mg. as compared with 67.0 mg. in the wild Norway and 65.6 mg. in the Alexandrine. The great discrepancy in size is even more striking in 350 gram animals; at this weight the left adrenal illustrated for the domestic Norway weighed 37.4 mg., while that of its wild counterpart weighed 108.0 mg. A similar difference in size is shown in the males.

Growth curves. Figure 2 shows the combined weight of both adrenals in relation to body weight in male and female rats of the domestic and wild Norways, and wild Alexandrines. Adrenal weights were obtained

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[4] Since this method gives relative volumes rather than weights, it had to be assumed, in reducing to a weight basis, that cortex and medulla had the same density.

for 65 male and 42 female domestic rats (weighing from 30 to 430 grams); 68 male and 79 female wild Norways (weighing from 50 to 580 grams); and 38 male and 31 female Alexandrines (weighing from 25 to 220 grams). The straight lines shown were then fitted to these data by the method of least squares.

The adrenals of the wild Norway and Alexandrine rats of both sexes weighed far more than did those of the domestic Norway, particularly in the higher body weight ranges. At 100 grams body weight the adrenals of the male wild Norway weighed twice as much as did
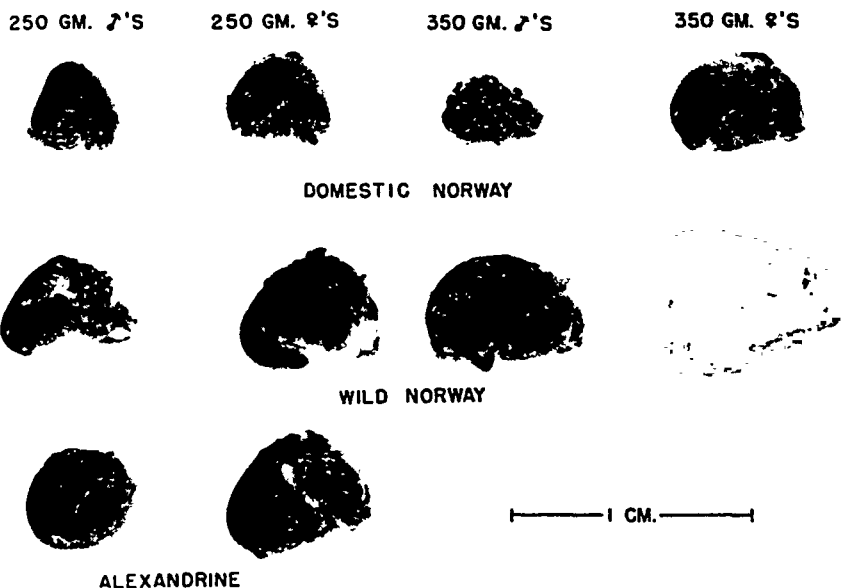
FIG. 1. Photographs of typical left adrenals of male and female domestic Norway, wild Norway and Alexandrine rats at two body weights.

those of the male domestic Norway and more than three times as much at 400 grams. The adrenals of the male Alexandrine were even heavier than those of the wild Norway at a corresponding body weight. There was no significant difference between the adrenals of the female wild Norway and Alexandrine.

Sex differences. Adrenal weights of male and female domestic Norway rats under 100 grams showed no marked differences (Fig. 2). Above 100 grams, however, the weights of the female adrenals were larger than those of the corresponding males. Thus, at 150 grams, the males had adrenals averaging about 25 mgs. and the females 40 mgs. At 300 grams body weight the males had glands averaging 38 mgs., while the glands of the female were twice that size.

This sex difference was also pronounced in the wild Norway rat. Figure 2 shows that, as in the domestic animals, adrenals were far

heavier in wild females than in wild males of the same body weight.
The divergence, however, starts at a lower body weight than in the
domestic animals. Thus, at 100 grams the glands weighed 65 mgs. in
females and 48 mgs. in males, while in the domestic rats the glands
weighed 27 and 22 mgs. respectively. At 400 grams the glands of the
wild rats weighed 250 and 143 mgs. respectively.

A   B
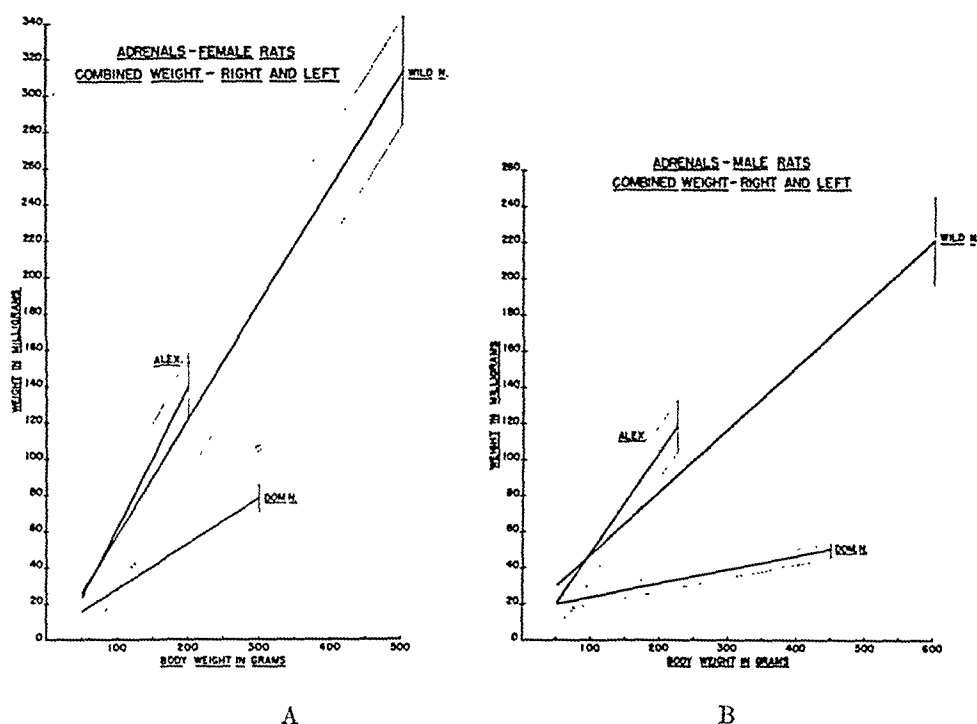
FIG. 2. Variation in weight of both adrenals with body weight for domestic Norway,
wild Norway and wild Alexandrine rats. Straight lines fitted to data by method of least
squares. Dotted lines (at plus and minus one standard error) indicate degree of scat-
tering around the lines: 68% of the individual values fall within the dotted lines in each
case. A. Female rats. B. Male rats.

In contrast, Alexandrine rats show little or no sex difference in
adrenal weights. Figure 2 shows that even in 200 gram rats, which are
large for their species, the females had adrenals weighing only slightly
more than those of the males.

Seasonal changes. The adrenals of wild Norway rats caught in the
warm summer months were smaller than those taken during early
winter. The seasonal difference is far more marked in the male than in
the female. Thus, in 350–400 gram rats the adrenals of males killed in
the winter months averaged about 50 per cent heavier than those
taken during the summer. In the females, the difference was too slight
to be statistically significant. Figure 2 gives data for rats caught
in the winter.

Domestic wild Norway crosses. Eleven male and 11 female offspring
from wild Norway rats bred to black domestic animals were employed

for this study. At the age of 6 months, when sacrificed, the 11 males weighed from 325–392 grams and the 11 females 215–275 grams. The average weight of the adrenals in the males was 61.2 mgs. or only slightly more than that of domestic males in the same weight group (40 mgs.). Corresponding wild Norway males, however, had adrenals weighing about 138 mgs. (see Fig. 2). The average weight was 75.0
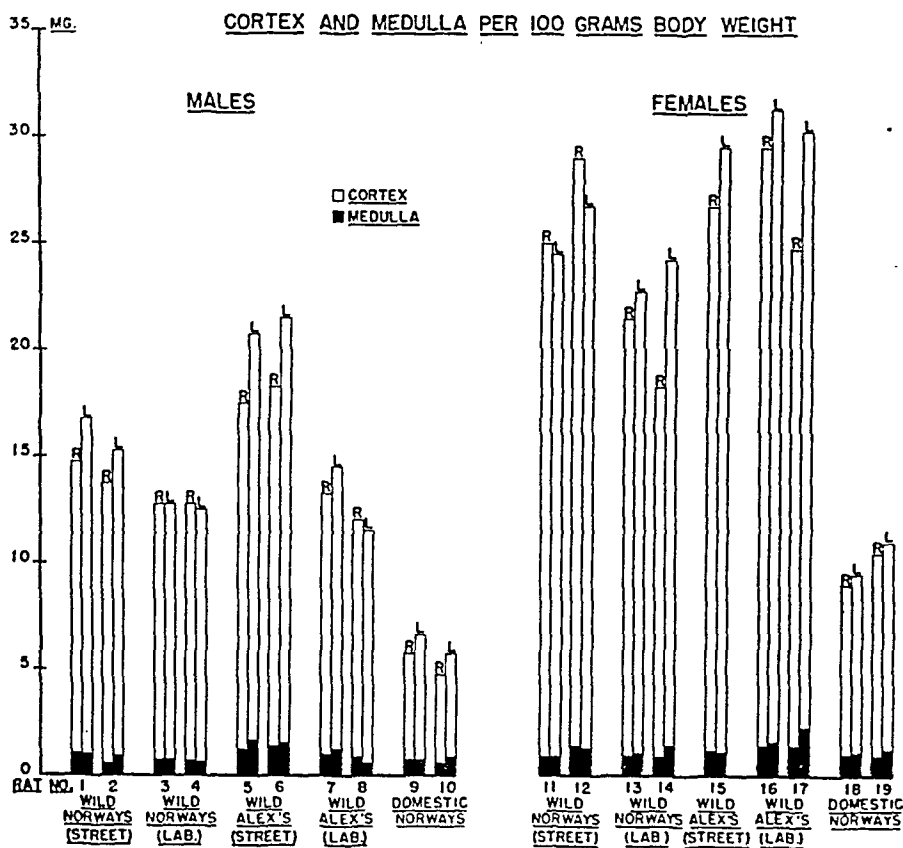
FIG. 3. Relative amounts of cortex and medulla found in the individual adrenals (left and right) of domestic Norway rats and of wild Norway and Alexandrine rats living under street or laboratory conditions. For purposes of comparison all data are given in milligrams per 100 grams of body weight.

mgs. for the females, compared with 64.0 mgs. for the domestic and 220.0 mgs. for the wild Norway females of corresponding body weight. Thus, the adrenal weights of rats of the first generation-crosses approximate those from the domestic rather than the wild parent.

Cortex-medulla relationship. Figure 3 gives the weights of the cortex and medulla, in mg. per 100 gram body weight, for both glands. This was determined in 19 rats by the combined histological and paper drawing methods.

The chart shows that a great reduction in the weight of the cortex accounts almost entirely for the smaller adrenal weights in the domestic rats as compared to either of the wild forms. In general the wild and domestic Norways had essentially the same medulla weights; the wild Alexandrines had heavier medullas. The females had larger medullas as well as cortices. The 12 month stay in the laboratory in general reduced the cortex weights, but the results are not consistent.

Histological differences. The adrenals of the wild rats had thicker fasciculata and reticularis. In all three kinds of rats the fasciculata was a slightly thicker band than the reticularis. The only marked cytological differences observed in the glands (regularly fixed in Bouin's solution and stained with hematoxylin and eosin) was in the juvenile cortex. However, no special fixatives or stains were used in this study. The fasciculata-like cells of the juvenile cortex, when present, were between the inner layer of the cortex and the medulla or embedded as extensions into the medullary material. These cells had about the size of the typical fasciculata cell but showed no evidence of vacuoles. The more usual arrangement was a tightly packed group forming either large or small islands in the inner region of the reticularis or between it and the medulla. This was true in both the street and laboratory wild Norways and to a lesser degree in laboratory Alexandrines. In the animals studied the islands of the latter group were infrequent and small. In contrast, the street Alexandrines, both male and female, showed a band of connective tissue, either wide or narrow, entirely surrounding the medulla. The juvenile cortical cells were embedded in this connective tissue band. In the case of the Alexandrine female the broad band of connective tissue and juvenile cortical cells was extremely vascular, far more so than the main part of the reticularis layer. These cells were present in incomplete bands between the cortex and medulla in the domestic Norway. However, the connective tissue, so striking in the Alexandrine, was entirely lacking.

DISCUSSION

The findings reported above demonstrate that one of the marked changes undergone by the Norway rat during its many generations of laboratory life, is a great decrease in the size of the adrenal glands in both sexes. Watson (1907) pointed out many years ago that wild rats after ten weeks of captivity had smaller adrenals than those killed in the field. Our study bears out this finding in that the adrenals of the Norways living under laboratory conditions were slightly smaller than those of rats fighting for their existence outside the laboratory, however, still not as small as adrenals from domestic animals. This decrease in the relative amount of adrenal tissue may be only an apparent one, owing to the fact that a wild rat living under laboratory conditions frequently accumulates fat. A larger quantity of fat would increase the body weight and so make the same size adrenals appear

to weigh less, when expressed per 100 grams of body weight. It is not known, however, whether or not this increase in fat is offset by an increase in muscular tissue in the street rat.

The great discrepancy in adrenal size is confined entirely to the cortex, as there is little or no change in the absolute amount of medullary tissue. Rats living a protected life in the laboratory where they are unaccustomed to great stress and shock apparently have no need for large amounts of cortical tissue. Griffiths (1944) has pointed out that domestic rats subjected to high frequency sounds have fits while the wild rats, both Norways and Alexandrines, are unaffected. We have found that while domestic rats survive adrenalectomy and, when given salt solution, continue to gain weight, wild Norways die within 12 days even when receiving salt in their drinking water or food (unpublished data).

The relation between the adrenal cortex and shock has been pointed out by numerous investigators who have shown that many forms of shock will cause adrenal hypertrophy. Short exposure to cold (Hartman, et al., 1931), stress (Ingle, 1943), vitamin E deficiency (Blumenfeld, 1934), morphine (MacKay, 1931), and numerous other drugs (Selye, 1937), cause an increase in the adrenal size of domestic rats. Adrenal hypertrophy is one of the changes taking place during the "alarm reaction" (Selye, 1936). However after shock the domestic rat adrenals never reach the size attained in the wild Norways. Selye (1937) reports a 50 per cent increase in adrenal weights of 90-day old rats after atropine, and MacKay (1931) has shown that acquired morphine tolerance will enlarge the adrenals as much as 70 per cent.

There is no evidence to show whether adrenal cortical hypertrophy is associated with hyperactivity. Flexner and Grollman (1939) have demonstrated that an increase in the cortical lipoid content of domestic rats tends to be correlated with greater activity but the complete "significance of the lipoid content of the cortex in relationship to functional activity is not definitely established" (Ingle, 1942). Further work must be done on the secretions of the cortical cells of the domestic rat and a comparison made between the quantitative output of these secretions and the activity of the two varieties of rats before it can be determined whether the decrease in cortical size is associated with a decrease in activity.

The medulla, in contrast to the cortex, does not appear to have changed significantly in size during the many generations of domestication undergone by the Norway rat, even though reduction in size of the cortex has given the medulla a greater proportion of the whole gland. On the other hand, in the Alexandrine rat we found medullary tissue in proportionately greater amounts than in the Norway. This fact may be related to the differences in behavior exhibited by these two species of rats.

While there was only a slight reduction in the weight of the adre-

nals of wild rats born in the laboratory, the F_1 generation of wild-domestic crosses had adrenals that were almost as small as those of the domestic. Thus, while in matings between wild rats many generations are needed to reduce the size of the adrenals down to the level of domestic rats, this result apparently can almost be achieved in crosses between wild and domestic rats in one generation.

It is probable that seasonal variations may be explained by the physiological reaction to the cold temperature of the winter months, the greater difficulty of obtaining food, and the general poorer health of street rats during the winter. Hartman (1931) and Selye (1936) have shown that short-term exposure to cold will cause adrenal hypertrophy. It has also been demonstrated that diet has a marked effect on the size of the adrenal (Blumenfeld, 1934; Ingle, 1945) and that unhealthy animals have larger adrenals than healthy ones (Grollman, 1936).

The islands of juvenile cortex seen in the wild Norways were similar in nature to those described in the domestic albino (Howard, 1938). The physiological significance of the band of connective tissue containing the juvenile cortical cells which surround the medulla of the wild Alexandrine is at present unknown.

SUMMARY

A comparative study was made of adrenal weights and cortico-medullary ratios in wild and domestic Norway rats and in wild Alexandrine rats.

In general the wild rats had proportionately much heavier adrenal glands than the domesticated form. Life under laboratory conditions from birth resulted in a slight reduction in adrenal size, but not as much as has occurred in laboratory rats after many generations of domestication. First generation crosses between wild and domestic Norways, however, had adrenals that weighed only slightly more than those of their domestic parents, indicating some genetic control of adrenal size.

The female Norway rats all had considerably heavier adrenals than the corresponding males. The sex difference in Alexandrines was not so marked.

A reduction in cortex size was found to be responsible for the difference in weight of the total glands, since the medulla was approximately the same size in all the rats.

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have small polymorphous nuclei ranging from round to flattened. The nuclei of the pineal cells are generally large and clear, and they possess several deeply stained, conspicuous nucleoli (figs. 1, 2 and 4). The nuclei of the pituicytes are smaller and more deeply stained and the nucleoli are inconspicuous (figs. 3, 13 and 18). Many of the nuclei of the pineal cells are bilobed, the two halves of the nuclei being separated by a deep narrow cleft or incisure which almost bisects the nucleus (fig. 4). A narrow bridge of protoplasm can usually be found connecting the two halves of the nucleus, although at times two separate nuclei are seemingly present. Occasionally one lobe appears to be much larger than the other and rarely 3 lobes are discernible. No similar configuration of the nuclei of the pituicytes has been encountered.

Nucleoproteins. Stained with methylene blue or eosin-methylene blue, the parenchymal cells of the rhesus monkey's pineal body possess a fairly abundant, moderately basophilic cytoplasm in which, with the oil immersion lens, minute particles and aggregations of basophilic material can be seen. In the neurohypophysis following the same procedure, little staining of the cytoplasm of the pituicytes can be made out; at most a little basophilic material is visible close to the nucleus. Although the pituicytes are sparsely provided with cytoplasmic basophilia, the surrounding neuropil shows a mild degree of basophilic staining. In the case of the application of eosin and methylene blue this manifests itself in a purplish shade.

When sections of the pineal gland are treated with ribonuclease before staining them with methylene blue or eosin-methylene blue, the blue-staining cytoplasmic component of the parenchymal cells is completely abolished (figs. 15 and 16). This loss of staining indicates that ribonucleoprotein is responsible for the cytoplasmic basophilia. The staining of the nuclei and nucleoli, on the contrary, is not very perceptibly diminished. After the Feulgen technique, the nuclear chromatin and a number of the nucleoli are stained intensely violet, a reaction which is regarded as being specific for desoxyribonucleoprotein. In addition to the several conspicuously violet nucleoli (karyosomes), a nucleolus can often be identified which is Feulgen negative and is tinged by the light green used as counterstain; this we interpret as being a nucleolus containing ribonucleoprotein.

The mild basophilia of the neurohypophysis which involves the neuropil and the cytoplasm of a few of the pituicytes is also prevented by ribonuclease (figs. 13 and 14). In other parts of the brain, especially in the grey matter, a similar staining of the neuropil with methylene blue is encountered.

Lipids. The staining of individual cells as observed in frozen sections of the pineal body and the infundibular process of the hypophysis is illustrated in figures 5 and 6. The parenchymal cells of the pineal body are rather uniform in appearance, exhibiting delicately

PLATE 1

FIG. 1. Pineal body of a young rhesus monkey. Fixation in Zenker's fluid followed by staining in eosin-methylene blue. Observe the basophilic staining of the cytoplasm of the parenchymal cells. ×10 oc., ×90 obj.

FIG. 2. Pineal body of a young rhesus monkey. Fixation in cold 80% alcohol followed by staining for alkaline phosphatase. Yeast nucleic acid utilized as substrate; section incubated for 72 hours at pH 9.5. Counterstained with paracarmine. ×7 oc., ×90 obj.

FIG. 3. Infundibular lobe of the hypophysis of a young rhesus monkey. Fixation in cold 80% alcohol, followed by staining for alkaline phosphatase. Fructose diphosphate utilized as substrate; section incubated for 72 hours at pH 9.5. Counterstained with paracarmine. ×7 oc., ×90 obj.

stippled grayish cytoplasm surrounding the nuclei (fig. 5). The parenchymal cells of the infundibular process, on the contrary, contain small, black sudanophilic bodies which range from none up to 20 or 30 in number (fig. 6).

Birefringence and autofluorescence. Frozen sections of the pineal body and neurohypophysis were examined for these properties. Excepting the collagenous fibers around the blood vessels which are always strongly birefringent, no doubly refractile substances are encountered in either the pineal body or neurohypophysis.

A very faint diffuse sheen is visible throughout the sections, but specific autofluorescence is not observed in the parenchymal cells. The elastic interna of the blood vessels fluoresces a luminous whitish blue wherever cerebral arterioles are seen.

In regard to these properties, the cells of the anterior lobe and pars intermedia of the hypophysis are negative throughout.

Glycogen. Following appropriate fixation of the pineal body and neurohypophysis in a mixture of absolute alcohol, formalin and picric acid followed by the Bauer-Feulgen stain for glycogen, cells and tissue elements of these organs are completely negative.

Upon applying McManus' periodic acid technique following the same fixative, several interesting observations can be made. The collagenous reticulum of both the pineal body and the neurohypophysis is stained a deep pinkish red which is not altered by previous exposure of the sections to saliva. In addition, some of the parenchymal cells of the pineal body show distinct red cytoplasmic staining. These are generally cells containing a relatively large amount of cytoplasm which is filled with minute red granules which are often arranged to form short rods or threads. In some few of the cells the reddish substance seems to extend out a slight distance into cytoplasmic processes of the cells. Other, more plentiful cells, exhibiting less cytoplasm, contain the reddish material only immediately around the nucleus, while still other cells which are quite numerous, contain none of these reddish elements whatsoever. Staining of the red substance is prevented by the use of saliva and consequently it must be attributed to the presence of glycogen.

It is interesting that glycogen does not become evident after the Bauer-Feulgen method but is visible only after the use of the periodic acid technique. The explanation which we have to offer is that the periodic acid procedure is very much more delicate than any of the previous methods for glycogen. We base this conclusion upon repeated comparisons of the results of the periodic acid method in a variety of tissues with Best's stain and the Bauer-Feulgen reaction as well as with the silver-nitrate method of Mitchell and Wislocki ('44). The periodic acid invariably brings out more glycogen than the latter and in the present instance it differentiates very delicate amounts of this substance where none is demonstrable at all by other histological means.

Another interesting feature of the periodic acid method is that it differentiates parenchymal cells in the pineal body, which contain glycogen, from others which contain little or none. This finding suggests that the parenchymal cells may be of different types or at least that they may be in different stages of functional activity. In the infundibular lobe of the hypophysis no glycogen comparable to that observed in the pineal body is encountered. In the adenohypophysis

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Explanation of figures on Plate 3

FIG. 7. Pineal body of a young rhesus monkey. Fixation in cold 80% alcohol, followed by staining for alkaline phosphatase. Fructose diphosphate used as substrate and section incubated for 72 hours at pH 9.5. ×240.

FIG. 8. Infundibular lobe of the hypophysis from the same monkey. Fixed and stained in the same way (fructose diphosphate at pH 9.5). Compare with field of the same seen at higher magnification in figure 3. ×240.

FIG. 9. Pineal body prepared with yeast nucleic acid as substrate and incubated for 72 hours at pH 9.5. Compare with field of the same seen in figure 2 at higher magnification. ×240.

FIG. 10. Infundibular lobe, the same as above, similarly stained with yeast nucleic acid as substrate at pH 9.5. ×240.

PLATE 3

no specific staining of the epithelial cells is noticeable by the periodic acid technique as we have used it. However, wherever colloid cysts occur in the adenohypophysis, the colloid filling these small vesicles reacts positively by both the Bauer-Feulgen and periodic acid techniques and in neither case is the staining prevented by saliva. The staining of this colloid by the Bauer-Feulgen method has been previously noticed by Wallraff and Beckert ('39).

Collagen. The staining of collagen and of collagenous reticulum in the pineal body and neurohypophysis has been observed in Masson preparations as well as in sections impregnated with silver by Pap's method ('29). The blood vessels penetrating the interior of these organs are surrounded by conspicuous sheaths or networks of reticular fibers (figs. 11 and 12). The vessels supplying these areas contrast sharply with the cerebral vessels penetrating the rest of the brain, which are not accompanied by any such elaborate and abundant connective tissue sheaths. The presence and permeability of these sheaths account apparently for the staining of the neurohypophysis, pineal body and other small areas of the brain by vital dyes such as trypan blue (Wislocki and King, '36; King, '39).

Nerve fibers. For the demonstration of nerve fibers the pineal body and neurohypophysis were stained by Bodian's protargol method following fixation of the tissues in Bodian's fixative no. 2. By this technique delicate nerve fibers are brought out in each area (figs. 17 and 18). In the case of the pineal body the fibers occur mainly on the outskirts of the parenchymal cords in association with the reticular networks subdividing the gland, as will be appreciated by comparing figures 11 and 17. The nuclei of the parenchymal cells are also quite intensely silvered. In the infundibular lobe, on the contrary, the fibers appear to be smaller, much more numerous and to run every which way without particular reference to any recurring pattern of the parenchymal cells or to the collagenous strands subdividing the infundibular process (cf. figs. 12 and 18).

Alkaline phosphatase. Both pineal body and neurohypophysis of the rhesus monkey contain alkaline phosphatase when sections are incubated at pH 9.5 for 24 hours or longer; after 3 hours' incubation

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Explanation of figures on Plate 4

FIG. 11. Pineal body of a young rhesus monkey, stained to show the argyrophilic collagenous reticulum. Fixation in a mixture of absolute alcohol, formalin and picric acid, followed by Pap's alkaline silver method for reticulum. ×140.

FIG. 12. The infundibular lobe of the hypophysis of the same monkey, similarly fixed and stained to show the argyrophilic collagenous reticulum. ×140.
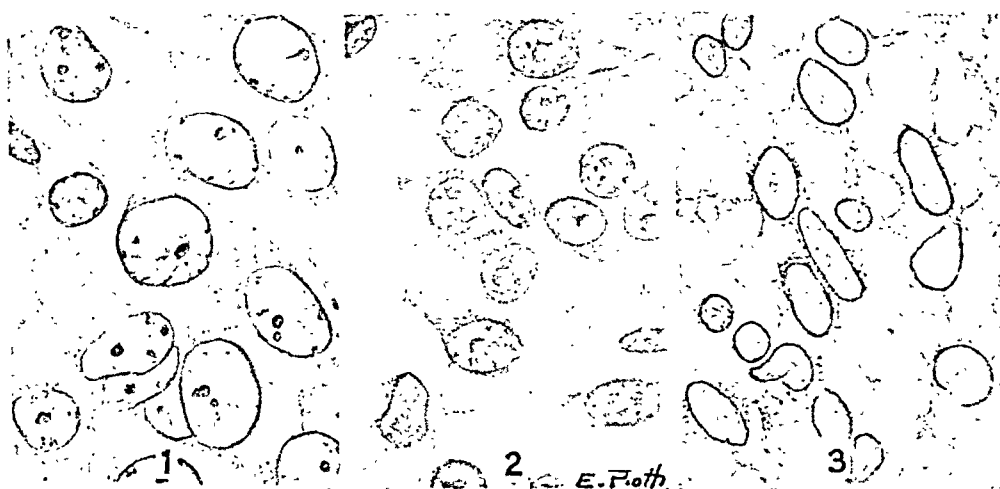
FIG. 13. The infundibular lobe of a young rhesus monkey. Fixation in Zenker's fluid followed by staining with eosin-methylene blue. Observe the relatively faint staining of cell cytoplasm and neuropil. ×220.

FIG. 14. The infundibular lobe of the same monkey, fixed as before. The section was exposed to a solution of ribonuclease before staining with eosin-methylene blue. By comparison with figure 13, it will be observed that little besides the nuclei is visible after treatment with ribonuclease. ×220.

11

12

14

PLATE 4

the reactions are so very slight that they might well be designated as negative. The pineal body reacts more uniformly and strongly than the neurohypophysis; the reaction in the latter is more intense in the center of the infundibular lobe than in the peripheral part. Of the 3 substrates used, the strongest reaction is obtained with fructose diphosphate and the next best with nucleic acid, while with glycerophosphate the least activity is demonstrable.

Parenchymal cells, blood vessels and the perivascular sheaths of the pineal body give pronounced reactions (figs. 2, 7 and 9). The reaction in the parenchymal cells involves both the cytoplasm and nuclei and extends out a short distance into the cell processes. With glycerophosphate the reaction is least intense. With nucleic acid the walls of the blood vessels are especially deeply stained, while the reaction of the perivascular sheaths is less intense; with fructose diphosphate vessel walls as well as the perivascular tissue are deeply colored.

The parenchymal cells in the interior of the neurohypophysis exhibit intense reactions at pH 9.5 after 24 hours or more incubation with fructose diphosphate and nucleic acid as substrates (figs. 3, 8 and 10), the color with the former being most concentrated in the cytoplasm and with the latter about equally distributed between cytoplasm and nucleus. The cortical zone of the infundibular lobe is much less intensely colored, and with glycerophosphate there is practically no reaction anywhere in the neurohypophysis. The penetrating blood vessels are less deeply and uniformly stained than in the pineal body. The neuropil, in the center of the infundibular process, comprising fibrillar and punctate interstitial material, is variably deeply stained.

It will be recalled that the pituicytes are believed to be related to neuroglial cells (Bucy, '32) more particularly to astrocytes derived from spongioblasts (Griffiths, '39). In view of this, it is of interest that the pituicytes contain alkaline phosphatase, whereas the neuroglial

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Explanation of figures on Plate 5

FIG. 15. Pineal body of young rhesus monkey. Fixation in Zenker's fluid, followed by staining with eosin-methylene blue. Observe the size and staining of the parenchymal cells and compare with figure 1 which presents the appearance of a field seen under higher magnification. ×240.

FIG. 16. Pineal body of the same monkey, fixed as before. The section was exposed to a solution of ribonuclease, before staining with eosin and methylene. Observe that the cytoplasmic basophilia (cf. figs 1 and 15) has been totally removed from the parenchymal cells by the enzyme leaving only the nuclei stained. The nucleoli have also retained their basophilia. ×240.

FIG. 17. Pineal body of another monkey fixed in a mixture of absolute alcohol, formalin and acetic acid (Bodian's fixative no. 2) and stained by Bodian's protargol method. Observe the fields of parenchymal cells surrounded by relatively acellular zones in which silvered nerve fibers are visible. The nuclei of the parenchymal cells are quite intensely silvered. Compare the size and number of the nerve fibers with the dense network of reticular fibers surrounding the fields of parenchyma (fig. 11). ×240.

FIG. 18. The infundibular lobe of the hypophysis of the same monkey, similarly stained by Bodian's protargol method. Note the greater wealth of nerve fibers and the smaller, less conspicuous cells than in the pineal body. Compare this preparation with figure 12 in which the argyrophil collagenous reticulum has been brought out. ×240.
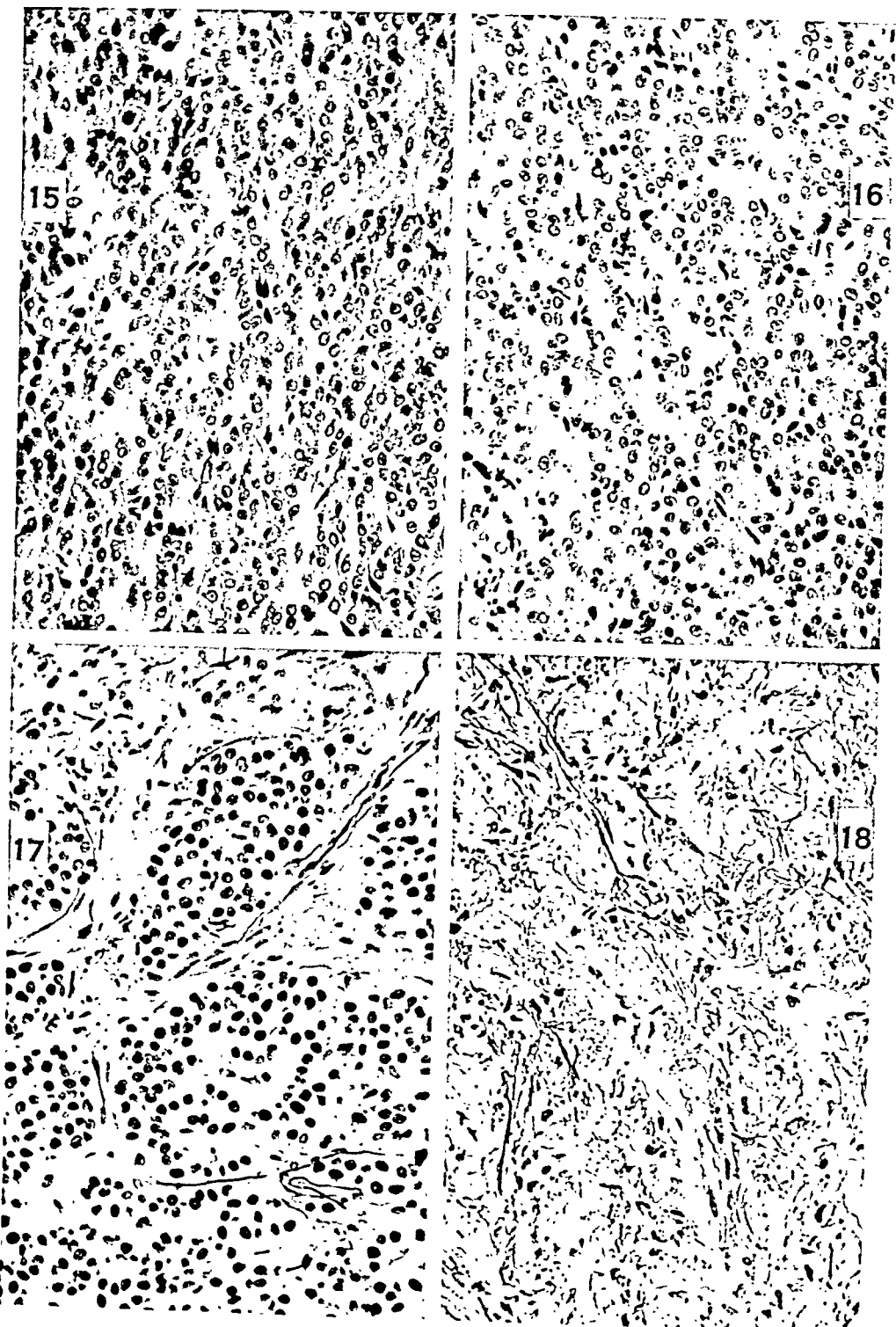
PLATE 5

cells, as observed throughout the cerebral convolutions in both grey and while matter, are very generally devoid of alkaline phosphatase in either cytoplasm or nuclei. In the cerebral grey matter we have observed repeatedly that alkaline phosphatase is most intense and most readily demonstrable in the blood vessels, that it occurs with great constancy in the neuropil as well as in the perikaryon and nuclei of the neurons, but that it is very nearly absent in the nuclei and bodies of the glial cells.

Incidentally to studying the neurohypophysis, the adenohypophysis has been examined. In general the latter is negative for alkaline phosphatase, although slight enzymatic activity is revealed in sections incubated for 72 hours utilizing fructose diphosphate and glycerophosphate. This little is present in quite irregular patches in the parenchymal cells of the pars distalis and pars intermedia. None has been encountered in the cells of the pars tuberalis, nor has any been seen in the walls of the blood vessels of any portion of the adenohypophysis. The absence of phosphatase in these blood vessels distinguishes them from the vessels penetrating the brain, the walls of which contain large amounts of alkaline phosphatase.

DISCUSSION

The present study reveals significant differences as well as some similarities in the cytology and histochemistry of the pineal body and neurohypophysis of the rhesus monkey. These will be briefly summarized and discussed.

Nucleoproteins. The parenchymal cells of the pineal body contain an appreciable amount of cytoplasmic ribonucleoprotein besides large and frequently bilobed, nuclei inside which are conspicuous chromatin bodies. The presence of large, chromatin-rich nuclei in combination with the basophilic cytoplasm suggests that the cells are engaged in active nucleoprotein synthesis. In possessing marked basophilia of both nucleus and cytoplasm they differ from the common neuroglial cells of brain tissue.

Some of the nuclei of the human pineal body contain conspicuous spherical inclusions (cf. del Rio-Hortega, '32) discovered by Dimitrova ('01). These bodies she regarded as liberating secretion, because at times, according to her, they approach the nuclear membrane, empty their contents into the cytoplasm and disappear. Basophilic granules which are said to be discharged into the cytoplasm have been described within them. Yet, Achúcarro and Sacristán ('12) have shown that the spheres of Dimitrova, instead of being nuclear, consist of cytoplasm enclosed within folds and inpocketings of the nuclei. Del Rio-Hortega ('32) believes, too, that the formation of the inclusions is initiated by depressions, wrinkles and folds of the nuclei. Besides the several authors who ascribe the role of nuclear secretion to these

structures, others have regarded them as representing regressive changes in the parenchymal cells.

In the pineal body of young rhesus monkeys we have not encountered any similar spherical inclusions. In view, however, of the deep incisure or cleft which exists in many cells between the lobes of the nucleus one can readily visualize how a pocket of cytoplasm might become sequestered and possibly completely snared off by progressive infolding of the nucleus.

To regard the spheres of Dimitrova as a part of the nucleus which discharges periodically into the cytoplasm is an interesting thought in view of the recent demonstration of Caspersson ('39) of the release of ribonucleoprotein into the cytoplasm by cell nuclei through the activity of the nucleoli. It seems likely, however, as del Rio-Hortega and others maintain, that these structures are cytoplasmic rather than nuclear. Nevertheless, the peculiar bilobed condition of many of the parenchymal cells resulting in an increase in nuclear surface might be a sign of increased nuclear activity in Caspersson's sense. And, further, if the nucleus were to liberate ribonucleoprotein into the nuclear cleft, its accumulation in the cytoplasm there might conceivably result in the formation of a basophilic retention vacuole identical with the spheres of Dimitrova. So that looked at in a somewhat different light, through information on nuclear activity recently supplied by Caspersson, one might suggest that the spheres of Dimitrova represent possibly nuclear secretion which has been discharged into the cytoplasm of the nuclear cleft.

In contrast to the parenchymal cells of the pineal body, the pituicytes of the neurohypophysis contain exceedingly little basophilic material in their cytoplasm and none of their nuclei exhibit distinctive clefts.

Lipids. Ignoring the faint grey stippling present in the background, pituicytes differ from the parenchymal cells of the pineal body in possessing definite lipid particles stainable by sudan black B. These bodies in the pituicytes of the monkey would seem to be homologous with similar sudanophilic lipid material discovered by Gersh ('39) in the pituicytes of a variety of mammals. Of the rhesus monkey, he speaks only of examination of a fresh, free-hand slice of the neurohypophysis in which the parenchymatous cells appeared as clear nuclei surrounded at times by fine, refractile granules. It seems likely that these granules would be homologous with the sudanophilic ones described in the present investigation. Gersh has suggested that this lipid is related to the production of the antidiuretic factor by the neurohypophysis, increasing in amount in the pituicytes when water-intake is restricted, but Hickey, Hare and Hare ('41) have denied such a relation. Gersh points out that cells bearing lipid material of this character are present only in the neurohypophysis among the

regions of the brain which he examined. He remarks that no cells of this type are encountered in either the area postrema or the pre-optic area, regions which, in respect to blood supply and some other histological features (Wislocki and King, '36), resemble the neuro-hypophysis. To Gersh's examples we can now add the cells of the pineal body as bearing no resemblance to pituicytes in regard to the possession of lipid particles.

Glycogen. The parenchymal cells of the pineal body contain saliva-soluble material in their cytoplasm, presumably glycogen, which is stainable by McManus' periodic acid method, but not demonstrable by the Bauer-Feulgen procedure. The periodic acid technique has revealed in our experience much more glycogen at known sites of its occurrence than any of the current methods for the staining of this substance. However, in the pineal body, small amounts of glycogen are revealed suddenly by this technique where none whatsoever is visible by other methods.

This is the only part of the brain among the areas examined in which periodic acid reveals any glycogen. None was encountered in the cells of the neurohypophysis, nor was any observed in neurons or neuroglial cells in white and grey matter in the hypothalamus or in areas adjacent to the pineal body.

Alkaline phosphatase. Phosphatases are enzymes capable of split-ting organic phosphates, for example, glycerophosphate, hexose diphosphate, nucleic acid and lecithin. They play important roles in the metabolism of carbohydrates, lipids and nucleoproteins. In the present investigation alkaline phosphatase has been found within the parenchymal cells of both the pineal body and the neurohypophysis. In the former it is associated with the presence of cytoplasmic nucleo-protein and glycogen, and in the latter with lipids which bear a pos-sible relationship to the specific functioning of the neurohypophysis. It is perhaps worthy of remark that, utilizing radioactive phosphorus in-jected intraperitoneally, Borell and Örström ('47) have found a high phosphate turnover in the pineal body of a variety of animals.

Concluding comment. The cytological evidence presented in this paper indicates that the cells of the pineal body and neurohypophysis differ very markedly, suggesting that their functional activities are probably very dissimilar.

Although the parenchymal cells differ in important cytological features, the similarity of the neurohypophysis and the pineal body in several histological respects is very close. It has been demonstrated (Wislocki and King, '36; King, '39) that the neurohypophysis, pineal body, area postrema and supraoptic crest have certain histological features in common which we shall briefly cite. (1) Their intrinsic blood supply is rich and the vessels are surrounded by conspicuous vascular sheaths composed of fibrous reticulum (cf. figs. 11 and 12). Both the pattern of the vessels and their connective tissue sheaths

distinguish them from the small cerebral vessels in general (cf. Cammermeyer, '44). This has been perhaps most conclusively demonstrated in the opossum where the neurohypophysis and area postrema are vascularized by plexiform blood vessels rich in connective tissue, whereas the rest of the brain is supplied by individual slender end-arteries and non-anastomotic, thin-walled capillary loops peculiar to the cerebral circulation of the opossum (Wislocki, '40). (2) These organs possess also in common the property of staining with vital azo dyes, a peculiarity which distinguishes them from the bulk of the brain which does not stain vitally because of the interposition of the "blood-brain" barrier (King, '39). (3) Finally, it might be pointed out that these structures are all in close proximity to the pia arachnoid, some, such as the neurohypophysis and pineal body, virtually protruding into the periaxial mesoderm. This consideration indicates again the peculiar association of these parts of the brain with connective tissue.

Because of these various properties, King has remarked that these structures "are in the nervous system but not of it." Closer perhaps to actuality would be to say that their specialized parenchymal cells are of the central nervous system, being related to and differentiated from ordinary neuroglia, but that the arrangement of their blood vessels and connective tissue as well as their permeability to vital dyes allies them to mesenchymal organs rather than to the brain. In the latter respect they are more like the peripheral nervous system or its derivative the adrenal medulla.

SUMMARY

The present cytological and histochemical investigations of the pineal body and neurohypophysis of the rhesus monkey reveal that the parenchymal cells of these two structures differ in a number of respects. The parenchymal cells of the pineal body are characterized by their large, chromatin-rich, lobed nuclei and the presence in their cytoplasm of ribonucleoprotein, alkaline phosphatase and traces of glycogen. The pituicytes, on the contrary, display lipid droplets, alkaline phosphatase, mere traces of ribonucleoprotein and no glycogen. These characteristics suggest differences in function in the two organs. Although the parenchymal cells of the pineal body and neurohypophysis are believed to be related to neuroglia, they have diverged from them to become separately specialized, judging from their histochemical properties.

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NOTES AND COMMENTS

CALCIUM DEPOSITION IN THE FEMORA OF CHICK EMBRYOS AFTER THE INJECTION OF ESTRADIOL BENZOATE[1]

It has been observed by many investigators that the injection of estradiol benzoate into various species of birds and mammals produces hypercalcification of the femora. The immature birds of some breeds show a more vigorous response than the adults (Day and Follis, 1941). However, in New Hampshire Red immature cockerels, the skeletal effects of estrogen are comparatively slight (Salomon, Gabrio, Reinhard, and Silberberg, 1947) while extensive hyperossification of the long bones occurs in the mature cock of this breed in response to estrogen (Landauer, Pfeiffer, Gardner, and Man, 1939). These and other variations suggest than an age factor partially determines the estrogenic response.

Fell and Robison (1934) studied histologically the development of the calcifying mechanism in chick embryos and reported that the osteoid tissue rapidly acquires a high degree of calcifiability. From about the 12th to the 21st days of incubation, fairly active bone formation is going on, and a relatively greater amount of calcium is available for bone calcification than in the adult stage.

From the foregoing considerations, it seemed of interest to investigate whether or not the hyperossification phenomenon could be produced at a very early stage of development and if estrogen could serve as an impetus to the calcifying mechanism or as a possible factor in the process. Thus, this experiment, which is preliminary in nature, was set up to determine if the injection of estradiol into the hen's egg prior to and after sex differentiation would result in hyperossification of the femora as indicated by increased calcium content.

MATERIALS AND METHODS

Fertilized eggs of New Hampshire Red Breed were used in this experiment. Various doses of alpha estradiol benzoate[2] were injected into some of the embryos under sterile conditions, and the eggs were returned to the incubator until the 15th or 18th day. At the time of sacrifice, the reproductive organs of the injected embryos and the controls were examined macroscopically.

Sixty-seven embryos were used in this investigation, and the 134 femora were analyzed for calcium content at the determined intervals. Ashing of the bones, prior to the determination of calcium, was carried out with concentrated nitric acid under an overhead heater (Nims and Horwitt, 1936)

and completed in a muffle furnace at 500°C. The ash was dissolved in 0.04 N hydrochloric acid and diluted with redistilled water. An aliquot was taken for calcium analysis following the titrimetric procedure of Salomon, Gabrio, and Smith (1946).

The chick embryos were divided into three groups:

Group I. Untreated Embryos. Since we were unable to find in the literature data pertaining to the calcium content of the femora of chick embryos at various stages of incubation, it was thought desirable to secure such information if possible. The femora of normal, untreated embryos and chicks were analyzed for calcium content at the following stages of development: 15 days incubation (7 females, 4 males), 18 days incubation (4 females, 6 males), and 1-day hatched chicks (3 females, 3 males).

Group II. Estrogen Injected at 10th Day of Incubation and Controls. Twelve embryos were injected on the 10th day of incubation with 1250 I.U. estradiol benzoate (2 females, 3 males), and 2500 I.U. estradiol benzoate (3 females, 4 males) respectively. Two female and 3 male uninjected embryos served as controls. All were sacrificed on the 18th day of incubation.

Group III. Estrogen Injected at 1st Day of Incubation and Controls. Sixteen 1-day embryos were injected with 1000 I.U. (4 females, 2 males), 2000 I.U. (2 females, 4 males), and 2500 I.U. estradiol benzoate (2 females, 2 males) respectively. Three female and 4 male uninjected embryos served as controls. All were sacrificed on the 18th day of incubation.

RESULTS AND COMMENTS

The analytical results obtained are represented in Tables I, II, and III. The data of Table I and the control data of Tables II and III indicate that no definite quantitative calcium content of the femora may be ascribed to a

TABLE I. CALCIUM CONTENT OF FEMORA OF UNTREATED EMBRYOS AND CHICKS

Group I	No. of femora	Dry wt. (mg.)		Mg. Ca.		% Ca. to dry wt.	
		Mean	σ	Mean	σ	Mean	σ
15-day Embryos	22	32.0	6.04	7.49	.930	24.03	4.345
18-day Embryos (Series A)	12	26.3	1.25	6.76	1.039	25.83	4.523
18-day Embryos (Series B)	8	32.2	4.42	12.14	1.322	37.97	4.029
1-day Hatched	12	58.6	4.93	11.53	1.222	19.69	1.714

particular incubation stage. For example, in the 18-day embryos of Table I, the eggs of Series A and the eggs of Series B were obtained from the same farm at different times, but analyses of the femora show great differences in calcium content. In the 18-day control embryos of Tables II and III still further variations are noted, so that there are four different mean values of calcium content of 18-day femora: 6.76 mg., 12.14 mg., 3.39 mg., and 3.03 mg. calcium respectively. The latter two mean values which are similar may be accounted for by the fact that the embryos of Tables II and III were hatched from the same batch of eggs. The many variable components such

TABLE II. CALCIUM CONTENT OF FEMORA OF 18-DAY EMBRYOS INJECTED
WITH ESTRADIOL BENZOATE ON 10TH DAY OF INCUBATION

Group II	No. of femora	Dry wt. (mg.)		Mg. Ca.		% Ca. to dry wt.	
		Mean	σ	Mean	σ	Mean	σ
1250 I.U. estradiol benzoate	10	16.7	3.50	3.36	.856	20.04	2.559
2500 I.U. estradiol benzoate	14	18.5	2.63	4.00	.540	21.87	3.534
Control	10	18.1	3.57	3.39	.645	18.96	3.032

as hen's ration, pre-incubation temperature to which the eggs were exposed, and the original weights of the eggs result in the extreme variations. However, the standard deviation of each group is small in comparison to the deviations between different groups. It is thus impossible to obtain satisfactory normal data on the calcium content of chick femora at different stages of incubation unless one considers a single series of embryos from one batch of eggs. Therefore, in this study of bone in response to estrogenic treatment in chick embryos, all of the groups were taken from a single batch of eggs.

The data of Table II indicate that neither 1250 I.U. nor 2500 I.U. estradiol benzoate injected on the 10th day of incubation caused increased amounts of calcium to be deposited in the embryonic bones by the 18th day of incubation. Since the standard deviations are comparatively large, the slight increases which may be noted in the treated embryos are without significance. Although Dantchakoff (1935) has reported that estrogen injection into 10-day embryos causes a slight degree of sex reversal in the males in some cases, we did not observe this phenomenon in any of our embryos injected on the 10th day, nor did Wolff and Ginglinger (1935) find intersexuality after the injection of folliculine on the 12th day of incubation.

From the data of Table III it appears that even though the embryos were exposed to the hormone for 17 days at the most, no significant change in calcium content of the femora can be noted in comparison to the controls. The injected females of this group did not show structural alterations of the gonadal system in response to estrogen except for one female who received

TABLE III. CALCIUM CONTENT OF FEMORA OF 18-DAY EMBRYOS INJECTED
WITH ESTRADIOL BENZOATE ON 1ST DAY OF INCUBATION

Group III	No. of femora	Dry wt. (mg.)		Mg. Ca.		% Ca. to dry wt.	
		Mean	σ	Mean	σ	Mean	σ
1000 I.U. estradiol benzoate	12	19.7	1.65	3.25	.286	16.53	1.043
2000 I.U. estradiol benzoate	12	19.0	1.59	2.97	.234	15.85	2.854
2500 I.U. estradiol benzoate	8	19.5	2.24	2.94	.335	15.06	.153
Control	14	21.9	1.45	3.03	.348	13.85	1.680

2500 I.U. estradiol and who exhibited a slightly lengthened right oviduct. However, in one of the males receiving 2500 I.U. estradiol, the left testis was flattened, larger, and composed of ovarian and testicular tissue (ovotestis). Remnants of oviducts persisted on both sides. In some of the other male embryos the gonadal changes were less pronounced. Our morphological findings then are similar to those of Willier et al. (1937).

Thus, while the injection of certain doses of estradiol benzoate into 1-day chick embryos affects the reproductive system by causing degrees of intersexuality, hypercalcification of the femora cannot be produced by this hormonal impetus. Although the calcifying mechanism of older birds is sensitive to estrogen, it seems that in the embryonic period a different process is in operation.

SUMMARY

Calcium analyses of the femora of untreated 15-day and 18-day embryos and 1-day chicks indicate that an acceptable series of normal chemical data cannot be obtained unless embryos from a single batch of eggs, hatched and kept under identical conditions, are used.

Calcium analyses of the femora of 18-day embryos, injected on the 10th day of incubation with 1250 I.U. and 2500 I.U. estradiol benzoate, indicate that hypercalcification of the femora does not occur in response to estrogen.

Calcium analyses of the femora of 18-day embryos, injected on the first day of incubation with 1000 I.U., 2000 I.U. and 2500 I.U. estradiol benzoate, also indicate that estrogen-induced hyperossification is not produced. However, some morphological changes in the gonadal system of the males were noted.

It is suggested that the response of the calcifying mechanism to estrogen is different in the embryonic period of chicks than the response at later stages of development.

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ENDOCRINOLOGY

METABOLISM OF THE STEROID HORMONES— THE METABOLISM OF PROGESTERONE AND ETHYNYL TESTOSTERONE[1]

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THIS INVESTIGATION is concerned with the possible conversion of ethynyl testosterone to pregnanediol-3(α),20(α), the question of absorption of progesterone from the gastrointestinal tract, and the conversion of progesterone to pregnanol-3(α)-one-20.

Allen, Viegiver and Soule (1944) reported the excretion of a conjugated steroid in the urine of patients suffering from secondary amenorrhea who had received ethynyl testosterone. Pregnanediol-3(α),20(α) was not actually identified but the authors suggested that the isolated material contained pregnanediol-3(α),20(α) or a related steroid. Other workers have been unable to demonstrate the presence of pregnanediol-3(α),20(α) after the administration of ethynyl testosterone (Goldberg and Hardegger, 1941; Hamblen, Cuyler and Hirst, 1940).

During the course of our investigations on the absorption of progesterone from the gastrointestinal tract a paper by Masson and Hoffman (1945) appeared which demonstrated that progesterone may be absorbed from the gut in the rabbit. Our studies on the absorption of progesterone from the gastro-intestinal tract were done in men.

SUBJECTS AND MATERIALS

The three subjects selected for these studies consisted of a thirty-two-year-old man (A. G.), who was suffering from Addison's disease

(Shipley, 1944), a seventy-one-year-old man (M. A.) who was diabetic and showed signs of hypopituitarism, and a 26-year-old woman (H. I.) suffering from secondary amenorrhea of about two years' duration.

Progesterone and ethynyl testosterone[2] were administered orally in capsules and the daily dose was usually administered in two equal doses in the morning and evening.

TABLE 1. THE EXCRETION OF PREGNANEDIOL AFTER THE ORAL
ADMINISTRATION OF PROGESTERONE

Patient	Clinical status	Experimental day	Amount of progesterone administered orally mg.	Pregnanediol-3(α) 20(α)[1] excreted in the urine mg./24 hrs.
A. G. (Male)	Addison's Disease	1	0	0
		2	0	0
		3	150	6.7
		4	0	0
		5	0	0
		6	300	21.1
		7	0	0
		8	0	1.6
M. A. (Male)	Diabetes	1	0	0
		2	0	0
		3	300	22.4
		4	0	13.7
		5	0	17.6
		6	0	1.3
		13	300	8.0[2]
		14	0	14.9
		15	0	10.1
		18	300	30.6
		19	300	45.0
		20	0	22.4
		21	0	13.9
		22	0	3.5
		23	0	1.6

[1] A portion of this was found to be pregnanol-3(α)-one-3 as described in the section, Conversion of Progesterone to Pregnanol-3(α)-one-20.

[2] Sixteen-hour sample.

The assays for pregnanediol glucuronide were done by the method of Venning (1938).

Studies on Ethynyl Testosterone

Ethynyl testosterone in total amounts of 600 to 1200 mg. was administered orally to all three of the subjects, A. G., M. A., and H. I. No pregnanediol glucuronide could be demonstrated in any of the urines collected after the administration of ethynyl testosterone.

Absorption of Progesterone

The gastrointestinal absorption of progesterone was studied in two male patients A. G. and M. A. Table 1 summarizes the experimental procedure and the results obtained. The percentage conversion of progesterone to pregnanediol appears to be lower in the Addison's disease patient than in the diabetic-hypopituitary patient. Thus in the Addison's disease patient the administration of 150 mg. of progesterone yielded only 4.5% pregnanediol, while 300 mg. of the hormone resulted in an excretion of pregnanediol only to the extent of 7.0%. In the second patient the administration of 300 mg. of progesterone per day resulted in pregnanediol excretions of 18.3% and 11.0% respectively. When 600 mg. of progesterone was administered, that is, 300 mg. per day for two days, the recovery for the period was 19.5%. Although the excretion product has been described as pregnanediol, it was demonstrated that a portion (approximately 10%) of the isolated product was pregnanol-3(α)-one-20.

Conversion of Progesterone to Pregnanol-3(α)-one-20.

The total amount of material designated as sodium pregnanediol glucuronide isolated from the urine of M. A. after the administration of progesterone was combined and amounted to 205 mg. of pregnanediol equivalent. This material was refluxed for six hours in a mixture of 200 cc. of distilled water, 30 cc. of concentrated hydrochloric acid and 300 cc. of benzene. The mixture was cooled, the benzene separated in a separatory funnel and the water layer reextracted twice more with 100 cc. portions of benzene. The combined benzene extracts were washed with water and evaporated to dryness in vacuo. The dry residue was treated with Girard reagent T and separated into the ketonic and non-ketonic fractions as previously described (Dorfman, Cook and Hamilton, 1939). The ketonic fraction yielded a crystalline material which after three recrystallizations from 90% methanol yielded a compound which melted at 143-146.5°C.[3] (weight 22 mg). This compound was mixed with an authentic sample of pregnanol-3(α)-one-20. (M. P. 145-148°C.),[4] and the melting point of the mixture was 144-146°C. An acetate was made which melted at 95-97°C. and when mixed with an authentic sample of pregnanol-3(α)-one-20 acetate (M. P. 96-97°C.) the melting point was 95.5-97°C.

DISCUSSION

Contrary to the findings of Allen *et al* (1944) we have been unable to demonstrate the presence of a steroid conjugate after the administration of ethynyl testosterone.

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[3] All melting points were taken with the Fischer-Johns apparatus and are uncorrected.
[4] We are indebted to Dr. K. Dobriner of Memorial Hospital for this sample.

There appears to be little doubt that progesterone is absorbed from the gastrointestinal tract. The recovery of as much as 18.3 and 19.5% of the progesterone as pregnanediol equivalent from the urine of one of our patients indicates that a good portion of the hormone was absorbed. However, these calculations are based on the assumption that the glucuronide isolated by the Venning procedure was pure pregnanediol. However, it has been shown that at least in one case the so-called pregnanediol glucuronide was not pure but actually contained pregnanol-3(α)-one-20 as an impurity. Thus if the yields are recalculated on the basis that progesterone was converted to both pregnanediol-3(α),20(α) and pregnanol-3(α)-one-20 it is found that 1.8% of the administered progesterone appeared as pregnanol-3(α)-one-20 and approximately 15.4% as pregnanediol-3(α),20(α).

The finding of pregnanol-3(α)-one-20 as a contaminant of the pregnanediol glucuronide preparation in this study is similar to the findings of Marrian and Gough (1946) who were able to demonstrate that so-called pure samples of pregnanediol glucuronide isolated from human pregnancy urines contained appreciable quantities of a water soluble complex of pregnanol-3(α)-one-20.

SUMMARY

It has not been possible to demonstrate the conversion of ethynyl testosterone to pregnanediol-3(α),20(α) or any other steroid either in men or in a woman suffering from secondary amenorrhea when daily oral doses of 300 mg. of the steroid were administered.

Progesterone absorption from the gastrointestinal tract was demonstrated by the isolation of sodium pregnanediol glucuronide after the administration of the progestational hormone. However, it was shown that the pregnanediol complex isolated by the Venning procedure contained a small amount of pregnanol-3(α)-one-20 in addition to pregnanediol-3(α),20(α). Thus it appears that pregnanol-3(α)-one-20 can be classed as a metabolite of progesterone along with pregnanediol-3(α),20(α).

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METABOLISM OF THE STEROID HORMONES— THE CONVERSION OF ISOANDROSTERONE TO ANDROSTERONE[1]

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THE FACT that the adrenal cortex contributes to the metabolic pool of androgens in the body and the 17-ketosteroids in the urine is well known. Hirschmann (1941) was able to isolate androsterone and etiocholanol-3(α)-one-17 from the urine of ovariectomized women in concentrations almost equivalent to that isolated from the urine of normal women. In Addison's disease the urinary titer of androgens and 17-ketosteroids is significantly reduced. In patients with adrenal cortical hyperplasia or tumors, the concentration of androgens and 17-ketosteroids is increased.

Although the evidence indicates that some of the androgens found in normal urine were derived from the adrenal cortical steroids, little is known of the mechanism of the conversion of these steroids to major androgen metabolites, such as androsterone and etiocholanol-3(α)-one-17. Of the three C-19 compounds which have been isolated from adrenal tissue, one, Δ^4-androstenedione-3,17, has been shown to yield androsterone and etiocholanol-3(α)-one-17 after administration to men (Dorfman, Wise, Shipley, 1947). However, it is to be remembered that Reichstein, who isolated these compounds from adrenal tissue, has indicated that these C-19 compounds may be artifacts.

Since a large number of compounds of the adrenal cortex have been shown to have the 3(β)-hydroxy-allo configuration in rings A and B and since only a small portion of the urinary steroids have this configuration, there appeared a good likelihood that compounds having this configuration may be further metabolized. Due to the fact that compounds of the adrenal cortical series having 21 carbon atoms were not available for metabolism studies, the model steroid isoandrosterone was investigated.

EXPERIMENTAL

The patient was a fifty-one-year-old man showing the symptoms of the Laurence-Moon-Beidl syndrome described elsewhere in de-

tail (Case 3) (Roth, 1946). The chief reason for choosing this subject was the fact that the individual has exhibited hypogonadism during his entire adult life. A control sample of urine collected over a thirteen-day period showed a 17-ketosteroid value of 3.2 mg. a day in the α-ketonic neutral fraction while the β-ketonic neutral fraction showed a titer of less than 0.05 mg. per day.

During the experimental period the patient received seven daily injections of 300 mg. of isoandrosterone acetate[2] contained in 6 cc. of sesame oil and an eighth injection of 40 mg. of isoandrosterone acetate. Urine was collected during the injection period and for the following two days.

The urines, both the control and experimental samples, were extracted daily after the completion of the twenty-four-hour period. For the extraction the urine was acidified with 15% by volume of concentrated hydrochloric acid and refluxed for ten minutes. The acidified hydrolyzed urine was cooled, 10 grams of sodium chloride was added per 100 cc. of urine and the mixture extracted with one-quarter volume of benzene. The benzene was separated and the extraction process repeated twice more. The combined benzene extracts were evaporated to dryness, taken up in ether and the ketonic neutral fraction prepared as previously described (Dorfman, Cook, and Hamilton, 1939). The nonketonic fraction was reserved for future isolation studies. The ketonic neutral fractions of both the control urine and the experimental urine were subjected to partition with digitonin, and the digitonin insoluble ketonic fractions and digitonin soluble ketonic fractions were prepared.

ISOLATION OF ISOANDROSTERONE: the digitonin precipitable ketonic fraction, which amounted to 0.2 gms., was adsorbed on a column of 5.0 gms. of aluminum oxide (Merck's Brockmann) from a 5 cc. solution of carbon tetrachloride. The column was eluted twice with carbon tetrachloride followed by carbon tetrachloride containing increasing concentrations of absolute ethanol ranging from 0.1% to 0.3% absolute ethanol in carbon tetrachloride, there was obtained 143 mg. of crystalline material, after recrystallization from methanol, which melted[3] at 173–174°C. A mixture of the crystalline material with an authentic sample of isoandrosterone (M. P. −172–3°C.) melted at 173–4°C. An acetate was prepared with pyridine and acetic anhydride which after recrystallization melted at 102.5–103°C. When mixed with an authentic sample of isoandrosterone acetate (M. P. 114.5–115.5°) the melting point was 112–113°C.

ISOLATION OF $\Delta^{2 \text{ or } 3}$-ANDROSTERONE-17 and ANDROSTERONE: The ketonic fraction which was not precipitated with digi-

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[2] We are indebted to Ciba Pharmaceutical Products, Inc., for the generous supply of isoandrosterone acetate.

[3] All melting points were taken with the Fischer-Johns apparatus and are uncorrected.

tonin amounted to 0.4 gms. It was subjected to chromatographic analysis using 12 g. of aluminum oxide. The urinary fraction was dissolved in 25 cc. of carbon tetrachloride and adsorbed on the aluminum oxide. Elutions were performed with 100 cc. portions of carbon tetrachloride and carbon tetrachloride containing 0.1% to 0.3% ethanol.

Elution with carbon tetrachloride (fraction 2) yielded 125 mg. of crystalline material which melted at 96–98°C. After four further recrystallizations from methanol the melting point was raised to 102–103°C. An oxime was prepared which melted at 152–153°C. When mixed with an authentic sample of $\Delta^{2 \text{ or } 3}$ androsterone-17 oxime (M. P. 151–2°C.) the melting point was 151–2°C.

One hundred and fifty-one milligrams of crystalline material was obtained from fractions 5, 6, 7, 8, which melted at 181–3°C. after recrystallization from methanol. When mixed with an authentic sample of androsterone (M.P. 181°C.) the melting point was 182–183°C. An acetate was prepared which melted at 162.5–163.5°C. When mixed with an authentic sample of androsterone acetate M.P. 160–160.5°C. the melting point was 160–161°C.

DISCUSSION AND CONCLUSIONS

The fact that isoandrosterone may be converted to and excreted as androsterone indicates a possible reason for the relatively high concentration of $3(\beta)$-hydroxy-allo compounds in the adrenal gland and the relatively low concentrations of these steroids in urine. In the metabolism of the adrenal cortical steroids to 17-ketosteroids, there still remains the consideration of the conversion of the C–21 steroids to the 17-ketosteroids. However, if this reaction does take place it can be seen that the $3\text{-}(\beta)$-hydroxy group can be inverted to the $3(\alpha)$ hydroxy configuration.

A likely mechanism for this inversion of the 3-hydroxyl group is by way of the 3-ketone with the subsequent reduction of the 3-ketone principally to the $3(\alpha)$ form. That such a reaction occurs is seen from the fact that after the administration of androstanedione $-3, 17$ the bulk of the 17-ketosteroids excreted is recovered as androsterone.

Seven and six-tenths per cent of the administered isoandrosterone was recovered from the urine as such while 8.1% appeared as androsterone. An additional 6.7% of the administered isoandrosterone appeared as $\Delta^{2 \text{ or } 3}$-androsterone-17. The latter compound may well be derived, at least in part, from androsterone. Thus the reported per cent of isoandrosterone (8.1) to androsterone is a minimum estimate.

SUMMARY

The administration of isoandrosterone to a hypogonadal man showing the signs of the Laurence-Moon-Beidl syndrome gave rise to the excretion of androsterone (8.1%) in addition to some unchanged

isoandrosterone (7.6%). An additional 6.7% of the administered isoandrosterone was recovered as $\Delta^{2 \text{ or } 3}$-androstenone-17. The latter compound may well be derived, at least in part, from androsterone.

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STUDIES ON THE BIOASSAY OF HORMONES— THE ASSAY OF ESTROGENS BY A CHICK OVIDUCT METHOD

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THIS COMMUNICATION is concerned with the response of the chick oviduct to various estrogens, particularly to the view of using this endpoint as a method of assaying estrogens.

ANIMALS, MATERIALS, METHODS

For these studies White Leghorn Pullets were employed which were obtained from Kerr Chickeries in Frenchtown, N. J. The animals were received in Cleveland between one to three days after hatching and immediately placed on a diet of commercial chick starting mash. The bulk of the experiments were started on the fourth day of life although some were started on the seventh day of life. In either case the material to be studied was injected subcutaneously once daily for five days and the determination of oviduct weight and body weight was done at autopsy 24 hours after the last injection. The total material administered per animal was dissolved in 0.5 cc. of corn oil and 0.1 cc. was injected daily. The unstimulated control group of animals received a similar schedule of treatment with only the corn oil.

The animals were killed with chloroform and the oviduct carefully dissected, blotted on a cloth towel and weighed immediately to 0.5 milligram on a torsion balance. All results are expressed as 100 times the ratio of weight of the oviduct in milligrams to the body weight in grams.

The estrogens studies consisted of stilbestrol, estrone, estradiol benzoate, estradiol, and methoxy bisdehydro doisynolic acid (MDDA).[1]

The method of studying the precision of the assay technique consisted in assaying an estrogen against itself using the design previously described by Bliss (1944). Thus the following experimental design was employed:

$$\frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown}}$$

and
$$\text{Standard} = \text{Unknown}$$

Using this design it was possible to easily ascertain the potency ratio, the error range of the potency ratio, and the significancy of the difference of the slopes.

RESULTS

Table 1 illustrates the crude data of the response of the oviduct to the various estrogens when the animals were autopsied on the 9th day of life.

In Table 1 we have 5 groups of animals which received no estrogen but did receive the usual amount of pure corn oil. The mean oviduct ratio varied from 9 ± 0.86 to 11 ± 0.87 for groups of animals which varied in number from 15 to 25. Stilbestrol which was the most active estrogen studied still showed a steeply rising curve when 1600 μg. total dose was administered. Thus the maximum dose level has not as yet been reached. However, at this level of 1600μg. of stilbestrol the mean oviduct ratio was 56 times as great as that of the controls.

TABLE 1. OVIDUCT RESPONSE TO ESTROGENS (9TH DAY)

Substance	Total amount admin. μg.	Number of chicks	Oviduct ratio, ±S.E.
0	0	15	11 ± 0.62
0	0	21	11 ± 0.87
0	0	25	10 ± 0.49
0	0	24	11 ± 0.43
0	0	17	9 ± 0.86
Stilbestrol	25	28	29 ± 1.8
	50	24	55 ± 2.3
	100	54	71 ± 2.4
	200	53	143 ± 6.3
	400	74	270 ± 10.3
	800	19	464 ± 29.4
	1600	18	564 ± 26.6
Estrone	2.5	16	12 ± 0.8
	10.0	21	14 ± 0.8
	40.0	18	16 ± 0.9
	160.0	52	20 ± 1.0
	640.0	68	31 ± 1.0
	1280.0	40	75 ± 1.1
Estradiol Benzoate	25	25	10 ± 0.5
	50	25	16 ± 0.9
	100	44	22 ± 1.1
	200	42	59 ± 3.8
	400	23	123 ± 8.6
Estradiol	100	34	26 ± 0.9
	200	30	33 ± 1.2
	400	52	41 ± 1.4
	800	21	69 ± 4.2
	1600	23	105 ± 7.1
MDDA	25	19	12 ± 0.8
	100	18	17 ± 3.0
	400	42	101 ± 28
	1600	45	108 ± 33

The response of the oviduct to MDDA is of interest from two points of view. First, although a reasonably good response of the oviduct was found at the level of 400 μg., increasing the dose to 1600

μg. caused no real increment in oviduct weight; a change from a ratio of 101 to that of 108. Secondly, as indicated by the standard error of the mean the variations of the responses within a group particularly at the levels of 400 and 1600 μg. are unusually great.

The Assay of Estrone

Tables 2 and 3 illustrate the use of the assay method for estrone. Table 2 is concerned with the data collected for the assays ending on the 9th day of life of the chicks while Table 3 is concerned with the data for the autopsies performed on the 16th day.

TABLE 2. ASSAY OF ESTRONE (OVIDUCT—9TH DAY)

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{1280 \ \mu g.}{640 \ \mu g.}$$

N	b	s	t	λ	Pot. Ratio ±S.E. %
5	150	8.87	0.531	0.059	83 ± 6
	156	16.3	1.542	0.104	117 ± 13
	127	18.9	1.567	0.149	127 ± 19
	154	25.6	0.228	0.161	107 ± 18
7	112	12.0	0.253	0.107	88 ± 7
	149	18.7	1.641	0.125	115 ± 13
10	140	18.1	1.880	0.129	112 ± 11
	153	15.4	0.935	0.101	100 ± 7
15	145	9.4	0.234	0.065	105 ± 4
	144	17.0	1.471	0.118	102 ± 7
20	147	16.7	1.040	0.114	105 ± 6

TABLE 3. ASSAY OF ESTRONE (OVIDUCT—16TH DAY)

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{640 \ \mu g.}{160 \ \mu g.}$$

N	b	s	t	λ	Pot. ratio ±S.E. %	Error range P = 0.05%
5	20.3	6.6	0.541	0.325	98 ± 33	−49; +95
	25.5	5.1	0.013	0.198	105 ± 21	−33; +51
	22.3	7.0	1.149	0.312	118 ± 38	−47; +90
7	20.6	6.2	0.457	0.302	101 ± 26	−41; +69
	23.7	5.0	2.810	0 211	113 ± 21	−31; +44
10	23.8	4.6	0.420	0.203	102 ± 15	−26; +34
	24.0	4.4	2.135	0.082	111 ± 15	−23; +31
15	22.7	5.2	1.069	0.228	107 ± 14	−24; +31

The tables list the values of N, b, s, t, λ, and the potency ratio ±S.E. N represents the number of sets at each dosage level, b the slope, s the standard deviation, t the significance of difference of the slopes, λ the ratio of s to b, and finally the potency ratio in terms of

the standard error of the potency ratio in percentage of original units employed for the potency ratio. To ascertain the total number of animals employed one multiplies the value N by four since each set contains 2 values on the unknown and 2 values on the standard.

The most favorable conditions for assay were found at the dosage levels of 640 μg. and 1280 μg. of estrone for the 9th day tests. Here the highest accuracy was obtained primarily due to the high value of the slope, b (table 2). In the 16th day tests, the dose levels of 1280 μg. and 640 μg. respectively showed the lowest error in the determination of the potency ratio. Actually the use of a total of 20 animals at 1280 μg. and 640 μg. will give a satisfactory assay using either the younger or older chicks. The younger animals showed a lower error in the potency ratio.

Of some 26 comparisons at the 9th day no instance of a significant difference in the slopes was found. Neither was a significant difference found in the slopes in any of the comparisons of the 16 day-old animals.

The Assay of Stilbestrol

All the animals were autopsied on the 9th day of life. The entire range of dosage levels from as little as 25 μg. total up to 1600 μg. yielded reasonably low error estimates with the lower doses having the higher reliability. Thus, using the range of 25 μg. and 50 μg. and a total of 20 animals a standard error of as little as ±10 per cent could be expected in the potency ratio (table 4). At the same dosage level an assay employing a total of 48 animals indicated a standard error in the potency ratio of ±7 per cent. In one instance of some 56 trials a significant difference in the slopes of the unknown and standard was found.

TABLE 4. ASSAY OF STILBESTROL (OVIDUCT—9TH DAY)

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{50 \ \mu g.}{25 \ \mu g.}$$

N	b	s	λ	t	Pot. Ratio ±S.E. %
5	98	6.46	0.066	0.413	98 ± 6
	91	11.7	0.129	1.900	96 ± 13
7	90	6.67	0.089	0.368	90 ± 17
	84	11.4	0.137	2.170	99 ± 12
12	90	9.92	0.110	1.252	100 ± 7

The Assay of Estradiol Benzoate

Table 5 illustrates the assay of estradiol benzoate. Only one set of concentrations is represented, that is, 100 μg. and 200 μg. Although the slopes varied considerably from 52 to 193 the values for λ were relatively constant. The variation in λ was from 0.082 to 0.116. The

errors of the potency ratio were quite reasonable. Thus using a total of 20 animals a mean standard error of the potency ratio (mean = 103) was only ±11 per cent. With the use of twice the number of animals in one trial run a potency ratio of 103 per cent was found with a standard error of ±7 per cent. No significant differences were found in the slopes of the unknown and standard.

TABLE 5. ASSAY OF ESTRADIOL BENZOATE (OVIDUCT—9TH DAY)

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{200\ \mu g.}{100\ \mu g.}$$

N	b	s	λ	t	Pot. Ratio ±S.E. %
5	72	7.2	0.100	0.589	100 ± 10
	52	7.7	0.148	0.349	107 ± 16
	167	13.7	0.082	1.755	101 ± 16
	193	22.4	0.116	0.518	105 ± 13
7	66	7.0	0.106	0.217	103 ± 9
10	180	17.7	0.098	1.429	103 ± 7

The Assay of Estradiol

Representative data on the estradiol assay are tabulated in Table 6. The range of 400 μg. to 1600 μg. total dose of estradiol was studied and a reasonably accurate determination of the potency ratio was found. The best precision was found between the levels of 400 μg. and 800 μg. of estradiol. When a total of 40 chicks were used at this dosage level the potency ratio of 77 ± 11 per cent was found in one trial and 101 ± 21 per cent in a second trial. The known potency ratio was 100 per cent. In one trial using a total of 48 chicks the value was 80 ± 11 per cent.

TABLE 6. ASSAY OF ESTRADIOL (OVIDUCT—9TH DAY)

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{800\ \mu g.}{400\ \mu g.}$$

N	b	s	λ	t	Pot. Ratio ±S.E. %
5	101	21.3	0.211	0.678	72 ± 17
	147	29.2	0.200	0.092	87 ± 18
	62	26.6	0.430	1.072	112 ± 54
	65.7	10.0	0.150	1.408	92 ± 14
7	86.5	20.4	0.236	1.245	72 ± 15
	121	26.4	0.218	0.586	84 ± 16
	65.2	21.3	0.327	1.242	103 ± 29
	69	10.0	0.145	1.260	85 ± 11
10	121	23.4	0.193	0.518	77 ± 11
	64	18.4	0.288	1.643	101 ± 21
12	111	24.1	0.216	0.873	80 ± 11

DISCUSSION

The data pertaining to the use of the oviduct as a method of assay of estrogen are presented in condensed form in Table 7. The value N is similar to that previously used in the paper, that is, N represents

TABLE 7. COMPARATIVE MEAN STANDARD ERRORS IN PERCENT
OF DETERMINATION OF POTENCY RATIO

(Numbers in parenthesis are number of trials)

Estrogen	Dose levels μg	Values of N					
		5	7	10	12	15	20
Stilbestrol	$\frac{50}{25}$	±10 (2)	±10 (2)	± 7 (1)			
	$\frac{200}{100}$	±16 (10)	±15 (2)	±11 (5)		± 9 (2)	
-	$\frac{400}{100}$	±20 (5)		±16 (3)		±13 (1)	
	$\frac{400}{200}$	±20 (5)	±15 (2)	±13 (2)		±11 (1)	
	$\frac{800}{400}$	±26 (2)	±21 (2)	±17 (N=9) (1)			
	$\frac{1600}{400}$	±31 (2)	±27 (2)	±20 (N=9) (1)			
	$\frac{1600}{800}$		±45 (2)	±14 (N=9) (1)			
Estrone (9th day)	$\frac{640}{160}$	±30 (4)	±22 (3)	±19 (2)		±12 (1)	±15 (1)
	$\frac{1200}{160}$			±20 (2)		±16 (1)	±14 (1)
	$\frac{1280}{640}$	±13 (4)	±10 (2)	± 9 (2)		± 5 (2)	± 6 (2)
Estrone (16th day)	$\frac{640}{160}$	±29 (3)	±22 (2)	±14 (2)		±13 (1)	
	$\frac{1280}{640}$	±14 (3)	±16 (2)	±14 (2)		±10 (1)	
Estradiol Benzoate	$\frac{200}{100}$	±11 (4)	± 9 (1)	± 7 (1)			
Estradiol	$\frac{800}{400}$	±29 (4)	±21 (4)	±16 (2)	±14 (1)		
	$\frac{1600}{400}$	±35 (2)	±39 (2)	±28 (1)			
	$\frac{1600}{800}$	±26 (2)	±25 (2)	±20 (1)			

the number of groups of four animals each. Thus the total number of animals is equal to N times four.

Although stilbestrol assays may be done at dosage levels of 25 μg. to 1600 μg. per animal the best accuracy was realized in the region of 25 μg. to 200 μg. The relative shallowness of the log dose-response curve of the latter region is more than made up by the low order of variability at any given dose level. The assay is quite remarkable when one considers that the use of only a total of 20 animals at the 25 μg. and 50 μg. levels could yield a determination of the potency ratio with a standard error of only ±10 per cent.

The bioassay of estrone can be accomplished with good precision by this method but unfortunately relatively large amounts of material are needed. This seriously limits the value of the assay. With a total of 20 pullet chicks at the concentrations of 1280 μg. and 640 μg. mean standard errors of the potency ratio of ±13 per cent and ±14 per cent respectively were found for the chicks autopsied on the 9th and 16th day. The best accuracy for estrone assay was found at the combination of the 1280 and 640 microgram levels using a total of 60 chicks. Under these conditions a standard error of the potency ratio of only ±5 per cent was found.

Estradiol benzoate could be assayed at relatively low concentrations with high precision. With the combination of 200 and 100 microgram levels of this estrogen ester using a total of 20 pullet chicks there has been realized a mean standard error in potency ratio of only ±11 per cent. Keeping all conditions constant but increasing the total number of animals to 40 resulted in a lowering of the standard error to ±7 per cent.

The choice of breed of pullet chick for the oviduct assay is presented in another communication (Dorfman, 1947). The question of diet should receive attention since it has been shown that at least one vitamin is essential for the responsiveness of the oviduct. The work of Hertz (1945) which has been confirmed in this laboratory indicates that in the absence of folic acid the chick oviduct shows only a limited stimulation even in the presence of adequate concentrations of estrogens.

SUMMARY

A new bioassay procedure for estrogens has been presented which although not possessing the advantages of sensitivity does have a high precision. The method consists in treating pullets with estrogens for five consecutive days during the first 15 days of life and determining the oviduct response 24 hours after the last injection.

Applications of the new assay procedure have been described with respect to stilbestrol, estrone, estradiol, and estradiol benzoate. The responses of the oviduct to MDDA (methoxy bisdehydro doisynolic acid) have also been described. The responses of the oviduct to MDDA

are unique since relatively small amounts produce good responses but. the log dose responses curve soon flattens out in spite of increased dosage.

At concentration levels of 25 μg. and 50 μg. of stilbestrol using a simplified statistical design, the potency ratio can be determined with standard errors of ± 10 per cent and ± 7 per cent respectively when totals of 20 and 40 pullet chicks were employed. Similar high accuracy was obtained for certain concentrations of estrone and estradiol benzoate.

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STUDIES ON THE BIOASSAY OF HORMONES— THE ASSAY OF PREGNANT MARE'S SERUM CHORIONIC GONADOTROPHIN

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This communication is concerned with studies on the bioassay of pregnant mare's serum chorionic gonadotrophin using the baby chick. Three endpoints have been studied. The oviduct response was found to be entirely unsuitable and the comb response of the male of doubtful value. However, the testis response appears to be a satisfactory endpoint having the advantages of reasonable sensitivity and reproducibility.

MATERIALS, ANIMALS, METHODS

The pregnant mare's serum hormone consisted of material kindly supplied by the Upjohn Company and the Schering Corporation. The material was readily soluble in water and was dissolved just before the first injections were done and kept at $-5°$ C. between injections. The total dose per animal was contained in 0.5 cc. of water.

The White Leghorn chicks were obtained from the Kerr Chickeries in Frenchtown, N. J., and usually arrived at the laboratory one day after hatching and immediately placed on a diet of starting mash and water.

For the oviduct studies pullet chicks were injected daily for 5 consecutive days starting with the fourth day of life and the injections continued through the eighth day. Twenty-four hours after the last injection the animals were killed with chloroform and the oviducts removed, blotted on a towel and weighed to the nearest 0.5 milligram on a torsion balance. The response was expressed as 100 times the ratio of the weight of the oviduct in milligrams to the body in grams.

The male chicks were studied in an analogous manner except that the combs and testis were removed at autopsy. The response of these organs also were expressed as 100 times the weight of the gland in milligrams to body weight in grams. One series of animals were used for the testis response to pregnant mare's serum hormone which were autopsied at 17 days of age. These animals received 5 injections of the hormone, one each day, from the twelfth through the sixteenth day of life. Autopsy was performed twenty-four hours after the last injection.

Statistical Calculations

The experimental design was similar to that described by Bliss (1944) for the assay of penicillin. By this method is was possible to calculate the potency ratio, the error of the potency ratio and finally the significance of difference of the slopes. The various symbols used are similar to those employed by Bliss (1944). Thus N represents the number of sets used. Each set represented 2 animals on the standard and 2 animals on the unknown. Thus the total number of animals is equal to four times N. The slope is designated as b, s represents the standard deviation, and t represents Fisher's t value, a measure for the significancy of the differences between slopes of standard and unknown.

Essentially the experimental design was such that the following conditions were established:

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}}.$$

The relationship tested was the logarithm of the dose versus the response. The exact formulations are not presented here but have been described by Bliss (1944).

<div align="center">EXPERIMENTAL</div>

Oviduct Response

Although a significant increment in the ratio of the oviduct was found with 50 i.u. of pregnant mare's serum hormone, quadrupling the dose to 200 i.u. caused no further increase in the ratio. The increase observed was of a low order, in the range of 28 per cent. On the basis of these poor responses no attempt was made to evaluate the method quantitatively.

Comb Response

The comb response was studied in male and female chicks. In male chicks about 40 i.u. of pregnant mare's serum hormone was required to produce a significant increment in the comb ratio. Be-

TABLE 1. THE ASSAY OF PREGNANT MARE'S SERUM HORMONE BY MEANS
OF THE COMB RESPONSE OF THE MALE CHICK

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}}$$

Unknown = Standard

(Chicks were 9 days of age at autopsy)

N	PMS conc. i.u.	b	s	λ	t	Potency ratio % ± S.E.	Error range P = 0.95
8	40; 62.5	84.0	21.5	0.256	0.493	81 +18	−36; +55
8	40; 125	62.8	22.8	0.364	0.975	165 ±50	−45; +83
8	62.5; 125	49.6	24.6	0.496	1.337	111 ±45	−56; +126

tween the levels of 40 to 125 i.u. a straight line relationship was found when the logarithm of the dose and response was considered. The equation for this relationship was $y = 50.4 \times -36.5$. In the female chicks as large a dose as 200 i.u. caused no real increment in the comb ratio.

Table 1 illustrates the use of the comb response as a method for the assay of the pregnant mare's serum hormone. Using 8 sets of animals or a total of 32 animals resulted in errors in the determination of the potency ratio ranging from -36 per cent and $+55$ per cent to -56 per cent and $+126$ per cent at $P = 0.95$.

Testis Response

A straight line realationship has been shown to exist between

TABLE 2. THE ASSAY OF PREGNANT MARE'S SERUM HORMONE USING THE
TESTIS RESPONSE OF THE CHICK

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}}$$

Standard = Unknown

(Chicks were 9 days of age at autopsy)

N	Conc. PMS i.u.	b	s	λ	t	Potency ratio % ±S.E.	Error range P = 0.95
14	25; 75	48.5	16.0	0.331	1.051	91 ±19	−34; + 50
8	25; 75	57.5	15.5	0.270	1.172	88 ±19	−36; + 55
		47.7	18.0	0.377	0.843	84 ±26	−46; + 85
8	62.5; 125	115	30.6	0.266	1.001	94 ±20	−35; + 55
8	62.5; 250	66.7	23.9	0.354	0.280	143 ±42	−45; + 81
8	125; 500	58.2	36.5	0.625	0.990	96 ±49	−64; +177
8	250; 500	98.3	27.0	0.275	0.053	133 ±32	−38; + 62
8	62.5; 500	77.5	18.4	0.238	0.286	134 ±26	−32; + 48

the logarithm of the dose and the testis response. The equation for the logarithm of the dose response relationship was found to be $y = 87.2 \times -94.9$.

From Table 2 it is seen that an accuracy of -34 per cent to $+50$ per cent can be realized in the determination of the potency ratio when a total of 56 animals are used. This was not much better than when only 32 animals were employed. In the dose range of 25 to 125 i.u. the error range was somewhat better than in the range 125 to 500 i.u.

One series of chicks were studied in which the hormone was administered once daily for 5 days starting with the twelfth day of life.

Autopsy was performed at 17 days of age. The control animals showed testis ratios considerably greater than was found for the animals autopsied at 9 days of age. Thus the younger animals showed a mean ratio of 18 ± 2 while the older animals showed a mean ratio of 31 ± 3. Further a striking difference was found in the relative sensitivities of the testis of the two age groups. As little as 25 I.U. of hormone caused a significant increment in testis mean ratio in animals autopsied at 9 days of age, an increase from 18 ± 2 to 37 ± 2. In the older animals the amount necessary to produce a significant increment in testis mean ratio was between 80 to 160 I.U. of the pregnant mare's serum hormone. The control mean ratio for the older animals was 31 ± 3 while 20 I.U. and 40 I.U. dosages produced mean ratios of 28 ± 1 and 30 ± 2 respectively. At 80 I.U. the mean ratio was 35 ± 2 and at 160 I.U. a mean ratio of 52 ± 3 was found. Using the Bliss (1944) design with the dosage levels of 80 I.U. and 160 I.U. a comparison was run comparing a standard and unknown where the standard was identical to the unknown. Using a total of 40 chicks a potency ratio of 113 per cent ± 20 per cent was found as compared to a theoretical 100 per cent. At $P = 0.95$ an error range of -30 per cent to $+42$ per cent was found.

CONCLUSIONS

The chick can serve as a suitable assay animal for pregnant mare's serum hormone if the testis response is employed. The comb size of the male has a more limited usefulness but the comb stimulation in the female was of no value under the conditions studied. The oviduct response was of no value. Thus the fact that only the smallest sort of stimulation of the oviduct could be demonstrated is not surprising since the relatively large amounts of estrogen that are required for oviduct stimulation (Dorfman, 1947). Thus about 40 micrograms of estrone and about 20 micrograms of estradiol are required to produce the stimulation found for 50 I.U. of pregnant mare's serum over the same period of time (5 days).

Of interest is the simultaneous increase in weight of the testis and the comb when stimulated with varying amounts of pregnant mare's serum hormone. Thus a straight line function for the testis growth was found from 25 to 500 I.U. without signs of the curve falling off. However, the comb growth increments seems to fall off beyond 62.5 to 125 I.U. This appears to indicate that certain nonandrogen producing elements of the testis continue to be stimulated. Or in other words it may be said that the androgen producing elements of the testis are quickly stimulated maximally.

A number of methods have been employed for the assay of pregnant mare's serum hormone. These consist of follicle stimulation, ovulation, corpus luteum formation, and estrus reactions in the vagina of the immature rat. No systematic statistical analysis for errors of

determination have been presented. Cartland and Nelson (1937) have used the ovarian weight of the immature rat as the endpoint for assay of the pregnant mare's serum hormone. The rats were Wistar strain animals weighing between 30 and 40 grams at 21 and 23 days of age. The hormone was administered in 3 daily doses and autopsy was performed 24 hours after the last injection. The aim of the assay was to produce mean pairs of ovaries equal to 65 mg. which was approximately 5 times the size of the ovaries of the normal untreated animals. By the use of 20 to 40 animals the authors claim that "this method has yielded reproducible results within an experimental error of ±10 per cent." No supporting data accompany this report.

SUMMARY

Pregnant mare's serum hormone can be assayed by means of the response of the chick's testis. Using a total of 32 animals (16 on the standard and 16 on the unknown) and using the experimental design of Bliss (1944) the potency ratio can be assessed with an error range of about −34 per cent to +50 per cent at $P = 0.95$.

The response of the chick's oviduct to pregnant mare's serum has been found unsatisfactory as an assay procedure. The use of the male chick's comb, but not the female chick's comb, is satisfactory for quantitative assay work. The comb test is inferior, however, to the testis test.

A much greater change in testis weight can be produced with increasing dosage of pregnant mare's serum hormone than can be produced in the comb. This seems to indicate that certain nonandrogen producing elements of the testis continue to be stimulated after the maximal response of the androgen producing elements have been reached.

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STUDIES ON THE BIOASSAY OF HORMONES—THE COMPARATIVE ACTIVITY OF ESTROGENS BY THE OVIDUCT RESPONSE IN CHICKS

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A METHOD employing the chick oviduct response previously described (Dorfman, 1947) has been employed to study the comparative activities of estrone, estradiol, stilbestrol, MDDA (methoxy bisdehydro doisynolic acid), and estradiol benzoate. As a result of an extended study of the increasing response with increasing dose of these compounds it was possible to pick regions of the respective log dose-response curves where the responses gave parallel lines for the evaluation of the comparative activities of various estrogens.

ANIMALS, METHOD AND MATERIALS

All chicks were White Leghorns and were supplied by Kerr Chickeries, Inc., Frenchtown, New Jersey. During the course of the investigations they were fed chick starting mash and water.

The standard procedure consisted of dissolving the test material in corn oil so that the total dose was contained in 0.5 cc. of oil. The material was injected subcutaneously once daily for 5 days (0.1 cc. of solution per day) from the 4th day of life through the 8th day of life. Twenty-four hours after the last injection the animals were sacrificed with chloroform and the body weights and oviduct weights were determined. All results are considered in terms of 100 times the ratio of the oviduct weight in milligrams to the body weight in grams.

The estrone, estradiol, estradiol benzoate, and MDDA were supplied by Ciba Pharmaceutical Products, Inc., and the stilbestrol was supplied by Wintrop Chemical Co., Inc.

The statistical calculations were patterned after Fischer (1934), Irwin (1937), and Bülbring (1935).

RESULTS

Table 1 lists the dosage levels studied and used for the calculations of the potency ratio, and the error range of the potency ratio.

Stilbestrol, MDDA, estradiol, and estradiol benzoate were found to have slopes which were not significantly different by the method

of Fischer (1934). In the case of estrone, the slope varied significantly from stilbestrol but not from that of estradiol benzoate. The relative potency of estrone in terms of stilbestrol was determined both directly and indirectly compared to estradiol benzoate. These considerations are listed in Table 2.

TABLE 1. RESPONSE OF CHICK'S OVIDUCT TO ESTROGENS

Compound	Dosage level μg.	Number of chicks	Mean oviduct ratio[1]	Standard deviation of mean
Stilbestrol	50	24	55	11
	100	54	71	17
	200	53	143	47
MDDA	100	18	17	12
	400	42	101	181
Estradiol	400	52	41	10
	800	21	69	19
	1600	23	105	34
Estradiol Benzoate	100	44	22	7
	200	42	59	25
	400	23	123	41
Estrone	640	68	31	9
	1280	40	75	7

[1] The ratio is 100 times the weight of the oviduct in milligram divided by the body weight in grams.

TABLE 2. THE RELATIVE POTENCIES OF VARIOUS ESTROGENS ON THE CHICK'S OVIDUCT

(Stilbestrol = 100 per cent)

Compound	Relative potency %	Error range % (P = 0.95)
Stilbestrol	100	—
MDDA	42	−29; +41
Estradiol	18	− 9; + 9
Estradiol Benzoate	48	−10; +10
Estrone	12 (12)*	− 7; + 7 (−5; + 5)

* This was found by comparing estrone to estradiol benzoate which was compared to stilbestrol in turn.

A further word may be mentioned about the activity of the estrogen MDDA. It will be noted that the standard deviation of the mean at both the 100 and 400 μg. levels is large as compared to that found for the other estrogens. This is due to the unusually wide dispersion of the individual observations.

DISCUSSION

This method affords a convenient and relatively accurate method for the studies on the comparative activity of estrogens. The fact that MDDA is assessed as 42 per cent of the activity of stilbestrol is a bit misleading. This relative activity only holds in the lower dosage ranges since further increases in dosage of stilbestrol results in great increases in the response of the oviduct. This is not quite true for MDDA since only a fraction of the animals show sizable responses at the higher doses.

Estradiol was found to be only 18 per cent as active as stilbestrol which is a rather low comparative activity. The increased activity of the estradiol benzoate is not unexpected since the standard method of administration consisted of one injection per day. The benzoate is well known as a derivative having a prolonged action.

Estrone has quite a low activity but of the same order as estradiol. Thus estrone was found to be 67 per cent as active as estradiol and only 12 per cent as active as stilbestrol.

Monroe and Kosin (1940; 1946) have used the chick oviduct method for the evaluation of the relative potencies of both naturally occurring estrogens and certain synthetic estrogens. The studies on the relative potencies of synthetic estrogens were done by incorporating the estrogen in the commercial chick starting mash at a level of 30 mg. of estrogen per pound of feed. By this method they found the following descending order of potency of six compounds as follows: dianisylhexane (dimethyl ether of hexestrol), dianisylhexene (dimethyl ether of stilbestrol), hexestrol, dienestrol, dianisylhexadiene (dimethyl ether of dienestrol), and stilbestrol. The studies of Munroe and Kosin (1940) on the naturally occurring estrogens using the chick oviduct response established the following descending order of activity: estradiol dipropionate, estradiol benzoate, estradiol, and estrone. No attempt was made, however, to quantitate the relative activities of the various estrogens studied.

SUMMARY

The comparative activities of four estrogens were studied using the oviduct response of the week-old White Leghorn chick. Using stilbestrol as the reference standard expressed as 100 per cent, MDDA (methoxy bisdehydro doisynolic acid) had a potency of 42 per cent; estradiol, 18 per cent; estradiol benzoate, 48 per cent; and estrone a relative potency of 12 per cent. The error range of the potency ratio of MDDA was found to be -29 to $+41$ per cent at $P = 0.95$ due to the high variation in the responses of the chick's oviducts to this estrogen. The other three relative potencies were determined with an accuracy of ± 10 per cent or better.

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STUDIES ON THE BIOASSAY OF HORMONES— THE COMPARATIVE OVIDUCT RESPONSE OF VARIOUS BREEDS OF CHICKS TO STILBESTROL

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IN PREVIOUS COMMUNICATIONS we have reported the use of the chick oviduct for the quantitative assay of such estrogens as stilbestrol, estrone, estradiol, estradiol benzoate and methoxy bisdehydro doisynolic acid (MDDA) (Dorfman, 1947A). This method was also employed to study the relative potencies of estrone, estradiol, estradiol benzoate, MDDA, and stilbestrol (Dorfman, 1947B). The question arises as to the relative sensitivity of various breeds of chicks to oviduct stimulation by estrogens. It was also of interest to determine the relative variation among individuals of the various breeds to the view of finding the most accurate conditions for the bioassay of estrogens.

ANIMALS, METHODS, AND MATERIAL

Four breeds of chicks, White Leghorns, White Rocks, Rhode Island Reds and a Sex Linked Cross (Plymouth Rock and New Hampshire Red) were obtained from Kerr Chickeries in Frenchtown, N. J.

The procedure consisted of dissolving the stilbestrol,[1] the estrogen chosen for all of these studies, in corn oil so that the total dose per animal was contained in 0.5 cc. of oil. The material was injected subcutaneously once daily for 5 days (0.1 cc. of oil solution per day) from the fourth day of life through the eighth day of life. Twenty-four hours after the last injection the animals were sacrificed with chloroform and the body weights and oviduct weights were determined. All results are considered in terms of 100 times the ratio of the oviduct weight in milligrams to the body weight in grams.

The calculations of the relative potencies (M), the standard error ($S.E.$) of the potency ratio, and the significancy of difference between slopes of the unknown and standard (t) were done by the method of Bliss (1944). The statistical calculations involving the relative sensitivities of the various breeds of chicks to estrogens were done by the methods described by Fischer (1934), Irwin (1937) and Bülbring (1935).

In the studies involving the determination of relative potencies the theo-

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[1] The stilbestrol was kindly supplied by the Winthrop Chemical Company, Inc.

retical potency ratio was always 100 per cent since the unknown and standard were identical.

EXPERIMENTAL

Table 1 illustrates the oviduct response of the four breeds of chicks to stilbestrol between the ranges of 50 μg. and 400 μg. of estrogen.

TABLE 1. OVIDUCT RESPONSES OF FOUR BREEDS OF CHICKS TO STILBESTROL

Breed	Total amount of stilbestrol administered μg.	Number of chicks	Oviduct Ratio $\pm$ S.E.
White Leghorn	0	21	11 $\pm$ 1
	50	24	55 $\pm$ 2
	100	54	71 $\pm$ 2
	200	53	143 $\pm$ 6
	400	74	270 $\pm$ 10
White Rock	0	12	20 $\pm$ 2
	50	21	57 $\pm$ 1
	100	22	86 $\pm$ 5
	200	20	156 $\pm$ 10
	400	22	239 $\pm$ 20
Rhode Island Red	0	10	17 $\pm$ 1
	50	19	47 $\pm$ 3
	100	20	84 $\pm$ 5
	200	20	174 $\pm$ 11
	400	17	290 $\pm$ 38
Sex Linked Cross (Plymouth Rock and New Hampshire Red)	0	12	17 $\pm$ 2
	50	23	43 $\pm$ 2
	100	21	103 $\pm$ 8
	200	22	209 $\pm$ 13
	400	20	284 $\pm$ 16

TABLE 2. COMPARATIVE REPRODUCIBILITY OF THE OVIDUCT ASSAY FOR STILBESTROL WHEN DIFFERENT BREEDS OF CHICKS WERE USED

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{200 \ \mu\text{g.}}{100 \ \mu\text{g.}}$$

(A total of 40 chicks were used for each comparison)

Breed	b	s	λ	t	M $\pm$ S.E.	Error range % (P = 0.95)
White Leghorn	391	56.9	0.145	0.322	105 $\pm$ 11	−19; +23
	334	72.4	0.216	0.183	88 $\pm$ 14	−27; +37
	300	41.5	0.138	0.995	93 $\pm$ 9	−18; +21
	205	22.8	0.111	0.246	105 $\pm$ 12	−20; +26
	204	21.2	0.104	0.226	100 $\pm$ 7	−14; +15
White Rock	233	37.3	0.160	1.031	83 $\pm$ 10	−21; +26
Rhode Island Red	596	33.0	0.055	2.341	112 $\pm$ 5	− 8; + 9
Sex Linked Cross	358	57.3	0.160	1.110	97 $\pm$ 11	−21; +26

The comparative reproducibility of the oviduct assay for stilbestrol using four different breeds of chicks is represented in Table 2. The concentrations of stilbestrol are 100 μg. and 200 μg. and the calculations are by the method of Bliss (1944). In each case the total number of animals used was 40 (20 chicks on the standard and 20 chicks on the unknown). The mean error range at $P = 0.95$ for the Rhode Island Reds are less than for the other three breeds.

Tables 3 and 4 further illustrate the comparative reproducibility of the oviduct assay for stilbestrol at 100 μg. and 400 μg. and 200 μg. and 400 μg. respectively. The error ranges found for the various breeds at 100 μg. and 400 μg. are quite similar. The White Leghorns, however, show a smaller error range at concentrations of 200 μg. and 400 μg.

TABLE 3. COMPARATIVE REPRODUCIBILITY OF THE OVIDUCT ASSAY FOR STILBESTROL WHEN DIFFERENT BREEDS OF CHICKS WERE USED

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{400 \ \mu g.}{100 \ \mu g.}$$

(A total of 40 chicks were used for each comparison)

Breed	b	s	λ	t	M ± S.E.	Error range % (P = 0.95)
White Leghorn	306	60.6	0.198	0.234	108 ± 16	−26; +35
	304	77.6	0.255	1.480	85 ± 16	−32; +46
	318	59.1	0.186	0.305	96 ± 13	−24; +31
White Rock	256	76.5	0.300	0.875	113 ± 25	−35; +55
Rhode Island Red	324	13.4	0.414	0.171	88 ± 27	+45; +82
Sex Linked Cross	306	97.5	0.316	0.761	70 ± 16	−37; +59

TABLE 4. COMPARATIVE REPRODUCIBILITY OF THE OVIDUCT ASSAY FOR STILBESTROL WHEN DIFFERENT BREEDS OF CHICKS WERE USED

$$\frac{\text{High Dose (Unknown)}}{\text{Low Dose (Unknown)}} = \frac{\text{High Dose (Standard)}}{\text{Low Dose (Standard)}} = \frac{400 \ \mu g.}{200 \ \mu g.}$$

(A total of 40 chicks were used for each comparison)

Breed	b	s	λ	t	M ± S.E.	Error range % (P = 0.95)
White Leghorn	391	56.9	0.145	0.322	105 ± 11	− 9; +11
	334	72.4	0.216	0.183	88 ± 14	−27; +37
White Rock	278	81.0	0.291	1.311	100 ± 21	−34; +53
Rhode Island Red	351	11.9	0.331	0.840	106 ± 24	−38; +62
Sex Linked Cross	253	98.5	0.389	1.390	78 ± 21	−43; +76

Comparative Oviduct Sensitivity of Various Breeds to Stilbestrol

Tables 5 and 6 illustrate the comparative sensitivity of the various breeds to oviduct stimulation by stilbestrol. Table 5 lists the regression coefficients calculated for the four breeds from the responses at 100, 200, and 400 g. of stilbestrol. From these data it was possible to quantitate the relative sensitivities which are presented in Table 6. The Sex Linked Crosses and the Rhode Island Reds appear to be the more sensitive. However, the White Leghorn and White Rocks have a sensitivity of about 77 per cent of the other two breeds.

TABLE 5. REGRESSION COEFFICIENTS CALCULATED FOR FOUR BREEDS OF CHICKS AT DOSAGES OF 100, 200, 400 G. STILBESTROL

Breed	Total number of animals	$\bar{x}$	Slope b	Constant a
White Leghorn	181	2.333	339	173
White Rock	64	2.301	255	167
Rhode Island Red	57	2.256	422	176
Sex Linked Cross	63	2.295	293	197

TABLE 6. THE COMPARATIVE SENSITIVITY OF VARIOUS BREEDS OF CHICKS TO STILBESTROL

Breed	Total number of chicks	Relative sensitivity %	Error range P = 0.95 %
Sex Linked Cross	63	100	—
Rhode Island Red	57	94	−15; +17
White Leghorn	181	77	−11; +13
White Rock	64	77	− 7; + 8

DISCUSSION AND CONCLUSION

These experiments were designed to answer two questions: A) What is the relative sensitivity of oviducts of various species to estrogens; and B) Is there any significant difference between the various breeds of chicks from the standpoint of overall accuracy for bioassay?

No great difference in oviduct sensitivity to stilbestrol was found for the four breeds studied. Actually the Sex Linked Crosses and Rhode Island Reds were the most reactive and no significant difference between the two breeds. The White Leghorn and White Rock breeds showed a significantly lower sensitivity. These breeds were found to possess 77 per cent of the reactivity of the Sex Linked Crosses.

From the standpoint of overall accuracy for bioassay there ap-

peared to be an advantage in using the White Leghorn breed. In a direct comparison between breeds the White Rocks, Rhode Island Reds, and the Sex Linked chicks gave a mean error range at $P \approx 0.95$ of -30 to $+43$ per cent; -30 to $+43$ per cent; and -33 to $+52$ per cent respectively, for a total of 40 chicks. Under similar conditions the White Leghorns gave a mean error range of -21 to $+28$ per cent.

SUMMARY

Four breeds of chicks, White Leghorns, White Rocks, Rhode Island Reds, and a Sexed Linked breed were studied with respect to oviduct sensitivity to stilbestrol. Rhode Island Reds and the Sexed Linked breed were the more sensitive. The other two breeds, the White Leghorns and White Rocks showed an oviduct sensitivity of 77 per cent of the Sex Linked chicks.

For use in bioassay the White Leghorn appeared to be the best from the standpoint of the lowest error range. Using a total of 40 chicks (20 on the unknown and 20 on the standard) the White Rocks, Rhode Island Reds, Sex Linked Cross, gave a mean error range at $P = 0.95$ of -30 to $+43$ per cent; -30 to $+43$ per cent; and -33 to $+52$ per cent respectively while under the same conditions the White Leghorns gave a mean error range of -21 to $+28$ per cent.

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THE ACCUMULATION OF RADIOACTIVE IODIDE BY THE THYROID GLAND IN NORMAL AND THYROTOXIC SUBJECTS AND THE EFFECT OF THIOCYANATE ON ITS DISCHARGE[1]

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INTEREST in the effects of thiocyanate on the thyroid gland followed the observation that goiter occurred in 11 of 246 patients treated with the material for long periods of time for hypertension (Barker, 1936; Barker, Lindberg, and Wald, 1941). Both the thyroid enlargement and the accompanying manifestations of hypothyroidism were relieved by thyroid administration. It was later postulated that the action of thiocyanate in producing goiter in rats differed from that of the thiouracil derivatives (Astwood, 1943). With the former the thyroid effect was much slower to develop and was completely abolished by added iodide, which did not modify the thyroid response to compounds of the thiouracil series. From studies of the accumulation of I^{131} by surviving thyroid slices from sheep, Franklin, Chaikoff, and Lerner (1944) showed that the iodide-concentrating capacity was inhibited by thiocyanate. Other goitrogens did not significantly diminish the accumulation of iodide by the tissue but prevented its conversion to diiodotyrosine and thyroxin. In intact animals, the administration of antithyroid substances caused the thyroid to enlarge and its iodide concentration to diminish (Astwood and Bissell, 1944). In such animals, however, the quantity of iodine remaining in the thyroid after treatment with thiouracil was found to be related to the iodine intake. If the iodine intake were greatly increased, large concentrations of iodine remained in the gland, but this iodine was shown not to be protein bound (McGinty and Sharp, 1946). Furthermore, the thyroids of rats depleted by thiouracil could entrap large quantities of injected iodide (Astwood, 1944–45). In thiouracil-treated animals, the entrapped iodide was spontaneously discharged so that about one-half was lost in five to six hours and a considerable portion of the remainder during the next eighteen hours (VanderLaan and Bissell, 1946). The *in vitro* experiments of Franklin, Chaikoff, and Lerner were confirmed by Wolfe, Chaikoff, Taurog,

and Rubin (1946), who showed that thiocyanate inhibited the accumulation of radioactive iodine by the thyroid gland of rats. Thiocyanate also strongly interfered with the accumulation of iodide by thyroids under the influence of propylthiouracil, provided the injected dose of iodine was small; with larger amounts, this inhibition was less striking (VanderLaan and Bissell, 1946). The iodine accumulated in the thyroid gland while under the influence of propylthiouracil was ultrafiltrable and behaved as iodide ion when studied potentiometrically using the silver-silver iodide electrode (VanderLaan and VanderLaan, 1947). These findings provided further evidence that the material was in the form of iodide. These same investigators found that thirty or more times the normal content of iodide was accumulated in thyroids made hyperplastic by propylthiouracil, even though the iodide concentration of the serum was not elevated. They further noted that thiocyanate not only prevented the uptake of iodide, but that it caused a rapid discharge of the iodide accumulated while the glands were under the influence of propylthiouracil.

The present report extends these studies by observations on normal human beings and subjects with hyperthyroidism without therapy and during the course of treatment with antithyroid drugs. It has been observed that a fully effective dose of an antithyroid compound given prior to the administration of a tracer dose of iodine profoundly modifies the course of iodine accumulation in the thyroid region. Under these circumstances radioactive material is rapidly concentrated in the thyroid gland, but while the total amount accumulated there is reduced, the maximal concentration is achieved in the brief interval of one to two hours. Thereafter the radioactive iodine slowly leaves the thyroid region; if the antithyroid treatment be maintained at a fully effective level, the iodine is completely lost from the gland in a few days. When the concentration of iodine in the thyroid is maximal, a large dose of potassium thiocyanate or of potassium iodide causes a rapid discharge of the radioactive iodine from the gland. The completeness of the disappearance of the previously accumulated radioactive iodine is related to the effectiveness of the antecedent antithyroid medication and to the dose of potassium thiocyanate or potassium iodide. Individuals suffering from hyperthyroidism exhibit a larger accumulation of iodide than normal persons, and the possibility of using this difference as a diagnostic method has therefore been tested. By using doses of potassium thiocyanate or potassium iodide which were found to be nearly completely effective, this phenomenon made it possible to study the effectiveness of the antithyroid pretreatment.

<center>METHODS</center>

Various amounts of different antithyroid drugs were ingested prior to the administration of the radioactive iodine in order to inhibit thyroid hor-

mone formation. In the studies designed to test the method as a means of diagnosis of hyperthyroidism, and in the several experimental procedures on normal subjects, it was desirable to attain as nearly complete inhibition of hormone formation as possible; this was accomplished conveniently by the oral administration of 100 milligrams of 2-mercaptoimidazole one-half to two hours before giving the I^{131}. When it was desired to estimate the degree of inhibition of hormone formation and, hence, the effectiveness of a therapeutic schedule in patients already receiving treatment, no additional antithyroid medication was given, the iodine being administered at the desired interval after the usual dose of drug. In all instances 100 microcuries of I^{131} without added carrier in weakly alkaline solution diluted to about 25 cc. with normal saline was given by mouth, followed by about 25 cc. of water to wash the material from the flask and the mouth and esophagus. Serial counts were then made at ten- to thirty-minute intervals over the isthmus of the thyroid gland with a shielded Geiger-Muller counter as described previously (Stanley and Astwood, 1947). When the accumulation of I^{131} in the thyroid region reached a maximum, as indicated by successive counts of the same magnitude, potassium thiocyanate, usually one gram, freshly dissolved in 25 to 50 cc. of water, was given by mouth. Counts were made frequently during the next hour to follow the discharge of I^{131} from the gland. The rate of loss was maximal during the first half hour after the ingestion of potassium thiocyanate and usually ceased during the second or third hour. The "background" was determined by counting over the left anterior chest at appropriate intervals during the procedure.

RESULTS

Normal Subjects: In 27 normal subjects after nearly complete inhibition of thyroid hormone formation by mercaptoimidazole the accumulation of iodide by the thyroid gland was slight, and the resulting curves quite unspectacular (Figures 1 and 2 and Table 1). The quantity of I^{131} accumulated was limited, and the maximum of less than 35 counts per second was reached in $1\frac{1}{2}$ to $2\frac{1}{2}$ hours. During the hour following potassium thiocyanate administration, the radioactive iodine was discharged from the thyroid at a rate similar to the rate of collection. The loss of I^{131} was frequently not quite complete at the end of the procedure, and counts were noted 2 to 4 counts per second higher over the thyroid than those of the general body background. Although these differences were quite small, they probably indicated significant concentrations of I^{131} in the thyroid gland, since after the administration of I^{131} to certain myxedematous patients, whose atrophic thyroids were incapable of accumulating the material, the radioactivity was found to be greater over the chest than over the thyroid region. On the other hand, in several of the normal subjects with very small uptakes of iodine, the radioactivity over the thyroid was less than background at the end of the test (Table 1) indicating that nearly complete discharge occurred under the influence of thiocyanate. When thyroid hormone formation was presumably completely suppressed by 100 milligrams of mercaptoimidazole and

the potassium thiocyanate was given *before* the I¹³¹, there was little or no collection of the radioactive iodine, by the thyroid gland as shown by the failure of the count over the thyroid to rise above that of the body background (No. 7, Table 1). In Figure 2 are shown the curves obtained after administration of I¹³¹ to two normal sub-
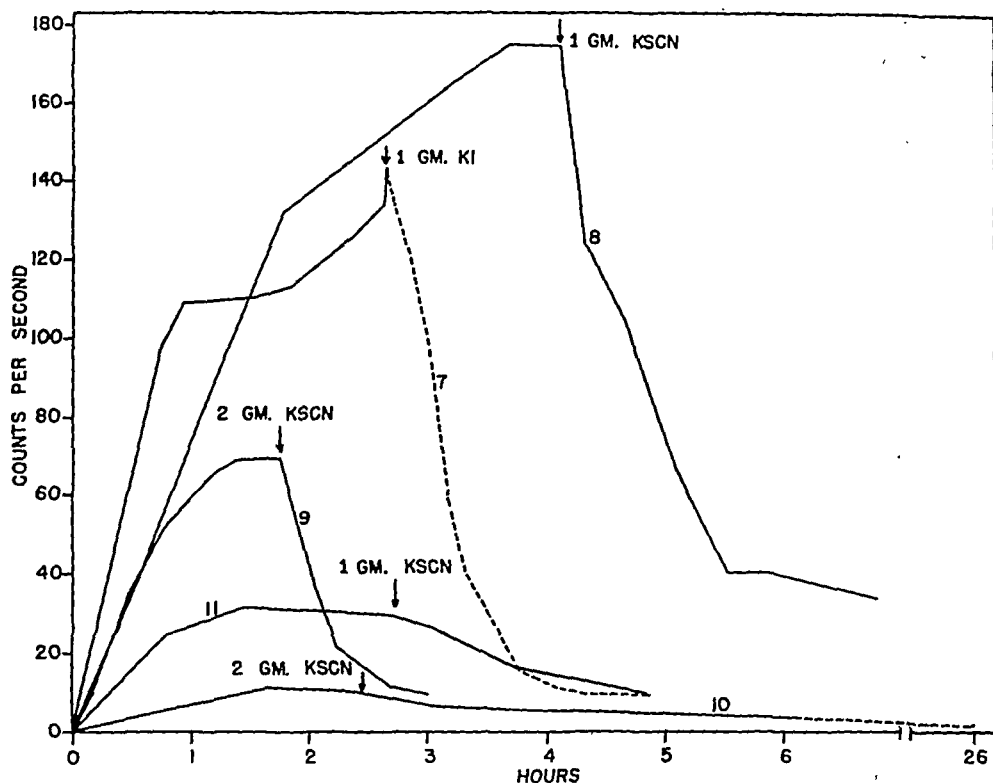
FIG. 1. The radio-iodide uptakes of two normal subjects (Nos. 10 and 11) and three patients with thyrotoxicosis (Nos. 7, 8, and 9). Each person had received 100 milligrams of mercaptoimidazole by mouth 65 to 80 minutes before administration of radioactive iodine to inhibit hormone formation. Nos. 8 and 11 took one gram and Nos. 9 and 10 two grams of potassium thiocyanate, while No. 7 was given one gram of potassium iodide, at the indicated times, also by mouth. Note the large iodide collection by the thyrotoxic patients as compared to the control subjects. When relatively complete inhibition was attained as in these individuals, the loss of the collected radio-iodide was more rapid than its accumulation in the thyrotoxic patients, while in the normals the rates of uptake and discharge were similar. The same data are given in Tables 1 and 2; the numbers are the same in the tables and in the above figure.

jects under the conditions described above, except that no potassium thiocyanate was given (see also Table 1). The rate of discharge of iodide was slow in all of the normal subjects; in those individuals with the smallest accumulation of I¹³¹ (maximal counts of 10 to 15 counts per second), there seemed to be little difference whether or not potassium thiocyanate was given, as the rate of discharge was similarly slow under both conditions. In three subjects no antithyroid agent was given before the radioactive iodine. The subsequent administra-

tion of 0.5 to 1.0 gram of potassium thiocyanate had little effect upon the course of iodine accumulation in the thyroid region and only a partial, transitory inhibition was observed. Doses of 10 milligrams or more of ordinary potassium iodide given after radioactive iodine merely caused the uptake of radioactive iodine by the thyroid to cease. Iodine which had already accumulated in the thyroid was not diluted out, indicating that it had quickly become bound to protein.

Thyrotoxic Patients: In each of 42 patients studied by a total of 90 tests (Figures 1, 2 and Table 2), the diagnosis of thyrotoxicosis was made on the basis of the usual clinical and laboratory grounds. No
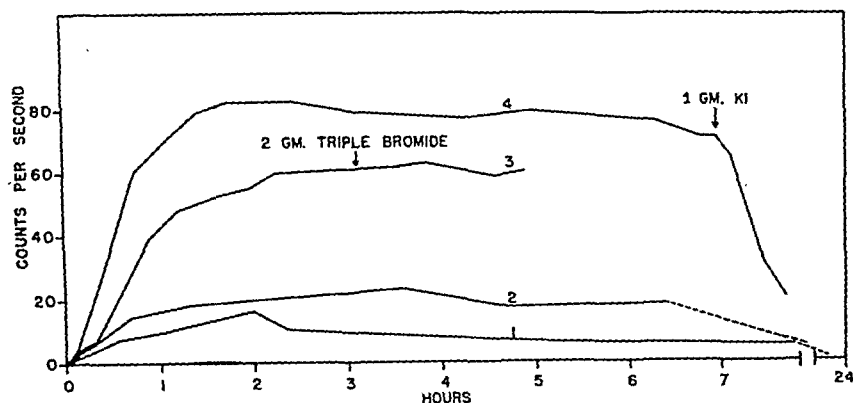
FIG. 2. The radio-iodide accumulation curves of two additional normal persons (Nos. 1 and 2), and two thyrotoxic patients (Nos. 3 and 4) under treatment with antithyroid drugs. All subjects had been given premedication to suppress thyroid hormone formation as indicated in Tables 1 and 2 (the numbers correspond to the same patients in the above figure and in Tables 1 and 2). These curves demonstrate the course of the radio-iodide levels in the thyroid gland when an inactive substance, such as the bromide ion (No. 3), was administered, or when no thiocyanate or ordinary iodide was given. It will be seen that the spontaneous loss of radioactive iodine was very gradual. In patient 4, one gram of ordinary potassium iodide produced the usual discharge seven hours after the tracer was taken. This indicated that the major portion of the I[131] was still in the form of iodide ion nine hours after the last dose of 20 milligrams of mercaptoimidazole. Presumably organic binding of iodide was effectively inhibited for at least this interval by this dose of mercaptoimidazole.

therapy of any sort had been administered prior to the test in ten patients (5 through 14, Table 2), with the exception of the 100-milligram dose of mercaptoimidazole given in all instances before the procedure to inhibit thyroid hormone formation. The pattern of accumulation and discharge of radioactive iodine was quite distinctive. There was a rapid uptake by the thyroid gland during the first one to two hours, with the level of the maximum higher than 60 counts per second in all cases. The rate of the spontaneous loss of the collected iodine was about the same as in the normal subjects (Figure 2 and Table 2). Within a few minutes after the administration of potassium thiocyanate by mouth, the radioactivity in the thyroid

decreased in a spectacular fashion, and the rapid discharge of accumulated iodide was usually completed in less than two hours. In most instances the count over the thyroid at the end of the test failed to reach the level of the background; this difference (2 to 24 counts per second) was usually greater than that which occurred in normal subjects. Too, when thiocyanate was taken before the I^{131} by thyrotoxic

TABLE 1. THE QUANTITY OF RADIOACTIVE IODIDE ACCUMULATED IN THE THYROID REGION IN 20 NORMAL SUBJECTS FOLLOWING THE ADMINISTRATION OF 100 MICROCURIES OF I^{131}. ALL SUBJECTS WERE TREATED WITH 100 MILLIGRAMS OF MERCAPTOIMIDAZOLE ONE-HALF TO TWO HOURS BEFORE THE TEST. THE MAXIMAL VALUE, THE TIME WHEN THE MAXIMUM WAS REACHED, AND THE EFFECT OF A SINGLE DOSE OF POTASSIUM THIOCYANATE ARE SHOWN IN SUBJECTS 7–20. IN SUBJECTS 1–6, THE SPONTANEOUS COURSE AFTER THE MAXIMUM ACCUMULATION AND WITHOUT THIOCYANATE IS DEPICTED

	Maximum accumulation	Time of maximum after I^{131} administration	Dose of KSCN	Course of I^{131} loss (No Thiocyanate) Time in hours after maximum					Background**
				1 hr.	2 hr.	3 hr.	4 hr.	24 hr.	
	c/s	Hours	Grams	c/s	c/s	c/s	c/s	c/s	c/s
1.	17	2:00	0	10	8	7	6	2	5–7
2.	23	3:34	0	18	18	18	—	5	5–7
3.	27	1:06	0	27	26	26	—	6	—
4.	15	:54	0	11	11	9	8	4	4–7
5.	12	:50	0	10	9	9	8	—	—
6.	19	2:23	0	17	16	16	17	—	—
			Course after KSCN						
7.	7	:48	1*	6	6	—	—	—	6–8
8.	9	1:09	2	5	4	4	4	1	4
9.	10	1:06	1	5	5	—	—	—	6–7
10.	11	1:40	2	6	5	4	3	—	4–7
11.	32	1:27	1	16	9	—	—	—	7
12.	21	2:22	2	13	8	—	—	—	5
13.	27	1:28	1	10	—	—	—	2	1
14.	33	1:28	1	10	6	—	—	—	4–5
15.	18	1:13	1	6	—	—	—	—	3–6
16.	20	1:10	1	10	8	7	—	—	4
17.	30	:42	1	8	6	—	—	2	—
18.	20	:56	1	13	9 (1:36)	—	—	—	5–7
19.	25	:56	2	9	—	—	—	—	6
20.	25	1:47	1	10	—	—	—	—	7

* This patient received the potassium thiocyanate 18 minutes before the I^{131} was given.
** Obtained by counting over the upper anterior chest.

patients (Table 2, No. 1), the count over the thyroid was not prevented from rising to a level which was above the background count.

  Determination of the Efficiency of Treatment with Antithyroid Compounds: The response to antithyroid medication in thyrotoxicosis depends upon such factors as the potency, the duration of action, the dosage and the individual sensitivity to the administered drug, previous iodine therapy, and the amount of hormone stored in the gland. Thus, evaluation of the efficacy of any compound is at best a lengthy process with many uncontrollable variables; at times correct judg-

ment of the efficacy of treatment by the usual clinical methods is impossible. The action of the thiouracil group of antithyroid drugs is a very selective one. These compounds cause an inhibition of the organic binding of iodine (which must first have been accumulated as iodide) to protein or in other words, these substances interfere

TABLE 2. THYROXOTIC PATIENTS: THE QUANTITY OF RADIOACTIVE IODIDE ACCUMULATED IN THE THYROID REGION IN PATIENTS WITH THYROTOXICOSIS WHO WERE GIVEN 100 MILLIGRAMS MERCAPTOIMIDAZOLE ONE TO TWO HOURS BEFORE THE TEST. THE EFFECTS OF IODIDE AND THIOCYANATE IN CAUSING LOSS OF THE COLLECTED RADIOACTIVE IODIDE ARE SHOWN IN DETAIL. PATIENTS 5 THROUGH 14 HAD NOT RECEIVED OTHER ANTITHYROID MEDICATION BEFORE THE TEST. PATIENTS 2 THROUGH 4 ARE INCLUDED TO SHOW THE SPONTANEOUS COURSE OF ACCUMULATED RADIOACTIVE IODIDE WITHOUT THE INFLUENCE OF THIOCYANATE. PATIENT 3 HAD 2 GRAMS OF "TRIPLE BROMIDES" WITHOUT ANY EFFECT ON DISCHARGE OF ACCUMULATED IODIDE

	Maximum accumulation	Time of maximum after I^{131} administration	Dose of KSCN or KI	Course of I^{131} loss Time in hours after maximum, or KSCN or KI administration							Background**
				½ hr.	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.	6 hr.	
	c/s	Hours	Grams	c/s	c/s	c/s	c/s	c/s	c/s	c/s	c/s
1.	17	:47	1* (KSCN)	16	13	—	—	—	—	—	6–11
2.	50	1:04	0	48	45	40	36	32 (3:42)	—	—	5–6
3.	62	2:14	0	60	61	60	60 (2:38)	—	—	—	8–10
4.	82	1:45	0	—	81	78	79	77	71 (5:11)	—	—·
	71	6:56	1 (KI)	33	21 (47 minutes)	—	—	—	—	—	—
				10 min.	20 min.	30 min.	1 hr.	2 hr.	3 hr.		
5.	60	1:34	1 (KSCN)	44	27	19	12	10 (1:30)	—	—	5
6.	102	2:54	1 (KSCN)	53	40	29	22	—	—	—	8
7.	143	2:20	1 (KI)	124	102	64	22	9	—	—	7
8.	175	3:40	1 (KSCN)	124	115	104	62	38	34 (2:38)	—	9
9.	70	1:52	2 (KSCN)	38	29	24	10	—	—	—	6–8
10.	79	1:16	1 (KSCN)	40	25	19	15	14	14	—	8
11.	90	1:18	1 (KSCN)	81	70	60	36	26 (1:49)	—	—	—
12.	90	1:10	1 (KSCN)	80	61	49	21	15	9	—	5
13.	95	2:23	1 (KSCN)	62	27	23	22	22 (1:33)	—	—	—
14.	69	1:19	1 (KSCN)	35	30	25	11	9	—	—	6–7

* This patient received an *additional* gram of potassium thiocyanate 33 minutes before the I^{131} was given.
** Obtained by counting over the upper anterior chest

with the first step in thyroid hormone formation. These drugs do not prevent the uptake of the iodide ion by the thyroid. Once bound to protein, thyroid iodine is no longer freely diffusible; it can no longer be discharged by thiocyanate or diluted out by ordinary iodide. Thus, the proportion of the accumulated radioactive iodine which leaves the thyroid under the influence of a standard dose of thiocyanate is an index of the degree of thyroid inhibition at the time of the procedure. At the end of the test the difference between the count over the thyroid region and the background represents radioactive iodine bound to protein during the period of the test. Presumably this iodine is available for the formation of thyroid hormone. These phenomena

can be directly utilized in determining the efficacy of antithyroid medication at any time after institution of therapy. By proper timing of the test in relation to the last dose, the duration of action of a single dose can be estimated.

In Figure 3 and Table 3 are shown examples of various degrees of inhibition of binding of accumulated radioactive iodine during therapy with antithyroid drugs. The significant findings here are the relative amounts of the accumulated iodine which failed to be discharged, and, hence, were presumably available for hormone synthesis. The absolute heights of the maxima were found to vary from time to time during treatment, but the factors involved in these changes are incompletely understood at present. The tests done after treatment in patient A (Table 3 and Figure 3) with propylthiouracil (300 milligrams per day) and mercaptoimidazole (40 and 60 milligrams per day) are good examples of the variations in degrees of inhibition of organic binding of iodine which may occur with different doses of antithyroid compounds.

Effects of Large Doses of Iodide and of Bromide Instead of Thiocyanate: If one gram of potassium iodide in solution were given during the test instead of thiocyanate, under like conditions of previous inhibition of hormone formation, the same distinctive pattern of discharge of acumulated radioactive iodine resulted (Figures 1 and 2). Bromide, however, failed to produce a similar effect in a dose of 2 grams of "triple bromides" (Figure 2).

Effects of Variations in the Administration of Thiocyanate and Iodide: Doubling the dose of potassium thiocyanate (2 grams in a single dose) in several instances accelerated somewhat the process of discharge of accumulated iodide, but it did not produce a detectably more complete loss of iodide from the thyroid gland (Figure 1). On the other hand, a dose of 0.1 grams of potassium thiocyanate produced an incomplete loss of iodide as demonstrated by a further discharge when a further dose of 1 gram was given. Similarly, the administration of only 10 milligrams of potassium iodide had no detectable effect in one subject. The ingestion of a second dose of 1.0 gram of potassium thiocyanate or potassium iodide during the one to two hours following the first dose of 1 gram of either occasioned a slight further discharge of iodide. These observations suggested that doses of 1 to 2 grams of potassium thiocyanate and 1 gram of potassium iodide were large enough for studies of this kind.

Determinations on the concentration of thiocyanate in the serum yielded values of from 3.3 to 9 mg./100 cc. (as potassium thiocyanate) during the interval of 14 to 63 minutes following ingestion of 1 gram of potassium thiocyanate. The data of Lavietes *et al.* (1936) showed that administration of 1 to 2 grams of thiocyanate consistently resulted in serum thiocyanate levels of 5 to 10 mg.%. A definite augmentation of effect was seen in one patient following administration
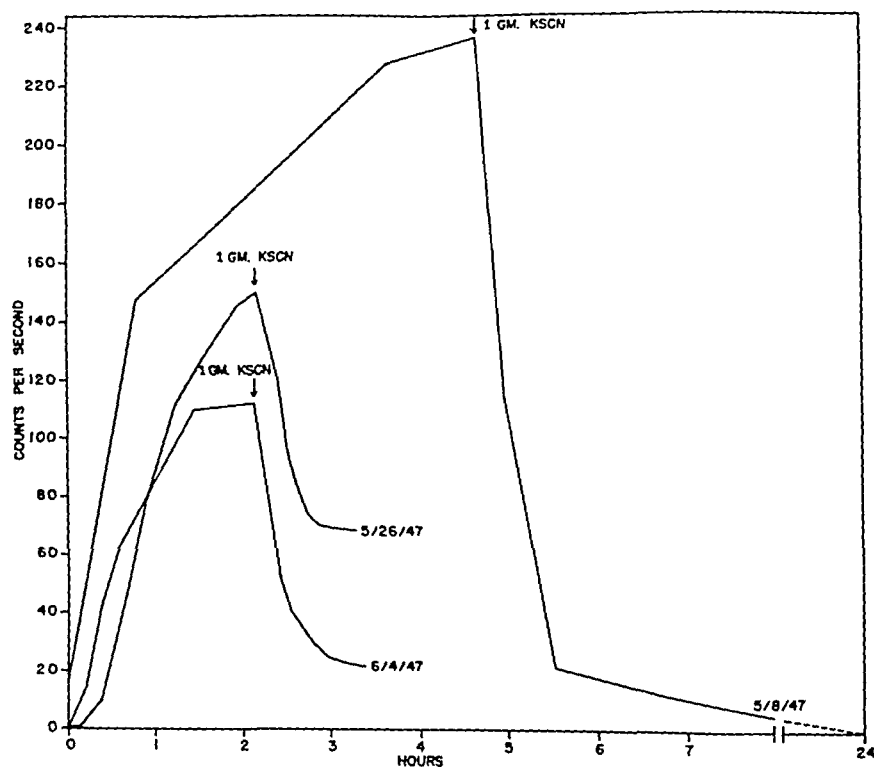
FIG. 3. The three graphs above represent radioactive iodide accumulations of patient A (Table 3) on three different occasions while she was receiving antithyroid drugs. The degree of inhibition of organic binding of collected radioactive iodine is indicated by the amount of iodide which was discharged by the potassium thiocyanate, relative to the amount accumulated before discharge began. On 5/8/47, with a dosage schedule of 20 milligrams of mercaptoimidazole every eight hours, there was only 2.3 per cent organic binding of collected iodide during the ten hours and twenty minutes' interval since the last dose (no further drug given during the test). While on propylthiouracil, 100 milligrams three times daily (test done 5/26/47), there was 45.3 per cent organic binding during the four hours and fifty-one minutes' period since the last dose of 100 milligrams. A third test (6/4/47), while the therapeutic schedule was 20 milligrams mercaptoimidazole every twelve hours, showed 21.3 per cent organic binding during the five hours and twenty minutes' period since the last dose of 20 milligrams. The values cited were all calculated from the level of discharge 59 minutes after the standard dose of potassium thiocyanate and hence are comparable, although they do not represent maximal loss in each instance. The control of the severe state of thyrotoxicosis as determined clinically could be correlated well with the above data. She seemed adequately treated with both 60 and 40 milligrams per day of mercaptoimidazole; she grew worse when 300 milligrams per day of propylthiouracil was being given.

of an additional gram of potassium thiocyanate when the serum level was 1.6 mg.%.

DISCUSSION

The rapid loss of radioactive iodine from the thyroid region produced by a large dose of non-radioactive potassium iodide suggests

TABLE 3. THE RESULTS OF TESTS FOR DETERMINATION OF THE EFFICIENCY OF INHIBITION OF ORGANIC BINDING OF IODIDE BY VARIOUS SCHEDULES OF TREATMENT WITH ANTITHYROID DRUGS IN TWELVE THYROTOXIC PATIENTS, AS DESCRIBED IN THE TEXT. THE COURSE OF THYROTOXICOSIS WHILE UNDER TREATMENT WAS ESTIMATED FROM CLINICAL AND OTHER LABORATORY DATA, AND WAS CORRELATED WITH THE RESULTS OF THE TESTS

Patient	Treatment	Maximal accumulation of I^{131} (c/s)	Interval in hours after last regular dose of antithyroid drug			I^{131} Remaining after KSCN (c/s)	% Uptake not discharged (organically bound)	Course under treatment
			When I^{131} given	When KSCN given	When test completed			
(A) 5/ 8/47	Mercaptoimidazole, 20 mg, every 8 hours for 8 days.	218.6	4:40	9:21	10:20 / 11:20	5 / 2	2.3* / 0	Prompt improvement in an extremely toxic patient Dosage reduced to 20 mg. every 12 hours 5 days later.
5/26/47	Propylthiouracil, 100 mg, every 8 hours for 7 days.	150.4	1:35	3:52	4:51	68.2	45.3	Grew worse when propylthiouracil substituted for mercaptoimidazole. Mercaptoimidazole, 20 mg, every 12 hours started again day of test.
6/ 4/47	Mercaptoimidazole, 20 mg, every 12 hours for 9 days.	112.5	2:15	4:21	5:20 / 5:38	24.1 / 21.3	21.3* / 19.0	Again improvement. It was repeatedly demonstrated that this dosage produced similar inhibition for as long as 9 hours. Clinical myxedema in 4½ months from beginning of treatment.
(B) 6/24/47	Mercaptoimidazole, 20 mg, every 12 hours for 14 days.	49.9	2:26	3:31	4:50	29.5	50.0	Moderate improvement but still toxic one month after this test; dosage then increased to 20 mg. mercaptoimidazole every 8 hours.
9/15/47	Mercaptoimidazole, 20 mg, every 8 hours for 7 weeks.	31.8	4:40	6:37	7:40	11.6	36.5	Early myxedema (total duration of treatment 3 months, 5 days).
(C) 8/13/47	Propylthiouracil, 50 mg, every 8 hours for 2½ months.	105.0	3:24	4:45	5:13	76.5	73.0	Still moderately toxic after 2½ months' treatment. Dosage increased to 100 mg. every 8 hours.
9/26/47	Propylthiouracil, 100 mg, every 8 hours for 44 days.	187.0	3:30	5:11	5:39 / 6:50	85.0 / 50.5	45.5* / 27.0	Much improved on increased dosage.
(D) 5/ 2/47	Propylthiouracil, 100 mg, every 8 hours for 3 weeks.	34.8	3:46	8:00	8:42	29.8	86.0	No improvement in symptoms.
5/16/47	Same dose total 5 weeks.	46.0	4:33	7:15	8:44	42.0	91.5	Still no improvement. Started on mercaptoimidazole, 20 mg, every 8 hours with rapid change toward euthyroidism.

Patient	Treatment	Maximal accumulation of I^{131} c/s	Interval in hours after last regular dose of antithyroid drug			I^{131} Remaining after KSCN c/s	% Uptake not discharged (organically bound)	Course under treatment
			When I^{131} given	When KSCN given	When test completed			
(E) 5/23/47	Propylthiouracil, 100 mg. every 8 hours for 6 weeks.	180.0	1:05	5:20	8:00	27.5	15.3	Rapid improvement from beginning of treatment. Early hypothyroidism after 6 months' treatment.
(F) 5/17/47	Propylthiouracil, 100 mg. every 8 hours for 9 days	91.7	2:53	4:42	5:32	37.2	40.5	Treatment started with mercaptoimidazole, 4/13/37 to 5/8/47. Moderately better at this test. Further progress gradual. Hypothyroidism 6 months from institution of treatment.
(G) 6/23/47	Mercaptoimidazole, 20 mg. every 8 hours for 2 days.	104.5	1:30	3:55	4:30	51.0	26.2	Rapid and continued improvement. Dosage reduced to 20 mg. every 12 hours.
7/ 7/47	Mercaptoimidazole, 20 mg. every 12 hours for 2 weeks.	190.6	2:05	3:20	3:55 / 4:55	68.6 / 37.1	36.0* / 19.5	Improvement maintained on smaller dose.
(H) 9/20/47	Mercaptoimidazole, 20 mg. every 8 hours for 7 weeks.	30.4	4:42	5:48	6:52	8.3	22.8	Rapid improvement from start of treatment.
(I) 10/ 8/47	Propylthiouracil, 100 mg. every 8 hours for 5 months.	130.0	4:15	5:38	6:42	65.0	50.0	Slow improvement. Goiter and blood cholesterol of 312 present at time of the test, but no clinical signs of myxedema were present.
(J) 9/15/47	Mercaptoimidazole, 20 mg. every 8 hours for 1 month.	94.5	2:14	4:29	6:15	22.8	24.2	Rapid improvement.
(K) 7/17/47	Propylthiouracil, 100 mg. every 8 hours for 3 months.	124.0	2:12	4:17	5:03	56.3	45.5	Improvement in symptoms during 3 months of treatment.
(L) 5/24/47	Propylthiouracil, 50 mg. every 8 hours for 4 months.	374.0	1:10	3:12	4:33	147.0	39.3	Improvement from 225 mg. daily maintained with 150 mg.

* Another test on same patient was terminated before maximal discharge had occurred. This value is for the concentration at the same interval after thiocyanate administration and allows direct comparison

that the iodine accumulated in the thyroid remains in the form of iodide ion when the gland is under the influence of an antithyroid compound. Apparently the thyroid gland possesses a mechanism by means of which the concentration of iodide ion in thyroid tissue can be maintained at a level many fold that normally present in the blood. In rats, VanderLaan and VanderLaan showed that this gradient of concentration was maintained despite a considerable increase in the total circulating iodine. They administered as much as 100 micrograms of potassium iodide without exceeding the capacity of the thryoid gland to maintain the same gradient. This dose corresponds to a dose of about 100 milligrams of potassium iodide to a human being. When a dose of 1 milligram was given to rats, corresponding to about 1 gram in man, only about one-tenth as high a gradient could be maintained. This probably indicates that there is a limit to the total amount of iodide that can be concentrated in the thyroid gland.

When a dose of 1 gram of potassium iodide is administered to man, the total iodide-ion in the body is greatly increased. The amount of iodide which the thyroid can accumulate is limited; hence, its ability to maintain a higher concentration in the thyroid than in the blood is markedly decreased in the presence of these large amounts of iodide in the body fluids. Since the specific activity of the iodide in the body fluids and the thyroid is the same, this sharp reduction in gradient is effective for the radioactive iodine atoms as well as the nonradioactive atoms; therefore, a much lower proportion of both remains in the thyroid, and the count over the thyroid drops rapidly as equilibrium is established.

The mechanism whereby thiocyanate acts is unexplained. One can only assume that it causes a breakdown in the selective mechanism for iodide concentration.

It is of some interest that virtually complete discharge of accumulated radioactive iodide was commonly observed in the normal individuals (i.e., count over thyroid was equal to or less than body background). This complete discharge did not occur in any of the thyrotoxic subjects with the exception of patient A (Figure 3 and Table 3, 5/8/47); the final levels were usually 2 to 24 counts per second higher than background. It was shown by VanderLaan and VanderLaan that a gradient of 250:1 for thyroid:serum iodide occurred in rats whose thyroids were hyperplastic from chronic propylthiouracil administration, whereas this gradient was only 25:1 in animals whose glands were normal. This ten-fold difference in concentration implies a still larger difference in total iodide, as the weights of the hyperplastic glands were several times greater than the normal weights. Thus, a difference of thirty-fold or more was observed when allowance was made for the larger size of the hyperplastic glands. Probably the effect of thiocyanate in one-to two-gram doses is to reduce greatly, but not to abolish, this gradient. If the gradient were

low in the beginning, as in normal subjects, thiocyanate might result in a near 1:1 thyroid:serum iodide ratio, whereas, if the gradient were very high, this same dose of thiocyanate would produce perhaps a 2-5:1 ratio.

There is an increased rate of production and secretion of thyroid hormone in thyrotoxicosis with a consequent increase in the turnover of iodine by the thyroid gland. Various components of this fundamental abnormality can be demonstrated with I^{131} in several different ways, including the method described in this paper. Another technique involves the administration of I^{131} without prior inhibition of hormone formation, the rate of accumulation being measured by counting over the thyroid region as described before. Rapid rates of uptake and large concentrations of I^{131} in the thyroid were encountered in previously untreated thyrotoxic patients, indicating that the major portion of the administered iodine entered the thyroid gland (Astwood and Stanley, 1947). The obvious explanation for this pattern of accumulation is that the I^{131} is entrapped as iodide by the thyroid cells which, being uninhibited, rapidly convert it into an organic form for storage in the follicles. Thus, the accumulation continuously increases because the I^{131} is not "dammed" in the thyroid cells in the form of iodide. However, the rate and extent of the accumulation in a few normal subjects and in some mild thyrotoxic patients fall within the same range. This overlapping detracts from the value of this method as a diagnostic procedure. A further disadvantage is that, in order to repeat the test during the course of treatment, therapy must be discontinued so that comparable results can be obtained, and no information as to the efficacy of treatment is obtainable.

A third variety of procedure involves the measurement of the I^{131} excreted in the urine during a period (usually 24 hours) following its administration (Rawson et al., 1944, 1945; Keating et al., 1947). The assumption is that the difference between the dose given and amount found in the urine reflects the quantity accumulated by the thyroid gland. The advantages of this type of test are that the estimations can be done at leisure, and precise measurements can be made of the quantity excreted. However, accurate urine collections are difficult unless the patient is under close observation in the hospital. A greater disadvantage is the relative paucity of information gained from the procedure; rapid changes in accumulation and discharge of iodine by the thyroid cannot be detected, and the method is inflexible.

If the rate of secretion of thyroid hormone could be measured, this would be a valuable aid in the diagnosis of thyrotoxicosis. Theoretically, this could be accomplished, using I^{131} as an indicator, by two methods:

In the first, the rate of loss of radioactive iodine from the thyroid gland could be determined. To do this the iodine could be permitted

to reach a maximal concentration and then observations could be made over several days while full doses of an antithyroid agent were given to prevent the reincorporation into the gland of I^{131} from catabolized thyroid hormone.

In the second method, the quantity of radioactive protein-bound iodine in the plasma could be measured following the administration of I^{131}. This method would require considerably larger, and perhaps dangerous, doses of tracer.

Both of these methods suffer the drawback of insufficient information to permit quantitative calculations. In order to calculate the actual rate of hormone synthesis or secretion, one would have to know the actual hormone content of the gland and the proportion of the radioactive iodine which was incorporated in the form of thyroid hormone.

The greatest single cause of error in these procedures for comparing the rates of turnover of thyroid iodine is the uncertainty in the previous iodine intake. As little as 3 milligrams of potassium iodide will greatly reduce the rate of uptake of a tracer dose of radioactive iodine by the uninhibited thyroid gland in a normal person. However, the quantity of iodide accumulated after a full dose of an antithyroid compound is much less affected by exogenous ordinary iodide. The finding of VanderLaan and VanderLaan that relatively large doses of ordinary iodide did not modify the thyroid to serum iodide ratio and the observation cited above that 10 milligrams of potassium iodide did not change the radioactive iodide concentration in man indicate that large differences in iodide intake would not give rise to error in this type of test.

The true significance of a large iodide accumulation by the thyroid gland is unknown. It is only known that a large iodide space develops in two conditions: in animals and man whose thyroids are made hyperplastic by prolonged antithyroid treatment, and in hyperthyroidism. Though hypertrophy and hyperplasia are common to these two conditions, it is not known that either of these states is directly related to the enlarged iodide space. Under most circumstances thyroid hypertrophy and hyperplasia are associated with depletion of colloid, a loss of total iodine, and an increase in vascularity. Further studies will be required to determine which aspect or concomitant of hyperplasia correlates with the enlarged capacity of the stimulated thyroid to concentrate iodide.

In any of the various tests discussed above, the administration of large amounts of iodine immediately prior to the test (either in the form of iodide or organically combined in compounds commonly used for x-ray visualization of the gallbladder, lung, or urinary tract) will often produce falsely negative results.

In following a number of patients during the course of therapy with various antithyroid compounds, the utility of the procedure

described herein for estimating the efficacy of treatment shortly after its inception was repeatedly demonstrated. If the level of I^{121} in the thyroid gland were very small after thiocyanate (less than 15 to 20 counts per second), indicating that nearly complete inhibition had occurred during the interval since the last dose of antithyroid drug, there was almost invariably a rapid response to treatment. This was particularly striking when the iodide uptake was very high, indicating that a large change had been effected in the originally great potential ability to produce hormone (patient A, Table 3 and Figure 3). This would be expressed both as a low percentage of the iodine used for hormone synthesis and a small absolute quantity remaining after thiocyanate. On the other hand, if the quantity of iodide accumulated in the thyroid were relatively low, as occasionally occurred while symptoms persisted (patient B, Table 3), and a small but significant amount remained after giving potassium thiocyanate, this prognosticated a poor response because only a small change in the rate of synthesis had been produced. This situation is expressed in Table 3 as a high percentage of organic binding and a low residual radio-iodine after thiocyanate. A large amount of I^{131} remaining after thiocyanate indicated a high level of organic binding, and hence, a suboptimal therapeutic response, even though this may represent moderate inhibition of synthesis originally extremely rapid (patient L, Table 3). Thus, one must take into account both the absolute level of iodine remaining in the thyroid gland after thiocyanate and the percentage inhibition of hormone synthesis which this level represents in order to anticipate properly the response to therapy.

Obviously, the test would be more informative if it were timed so that it ended when the next dose of drug was due; the inhibition of the thyroid would then be measured during the period of diminishing effectiveness and the result obtained in terms of minimal rather than maximal inhibition.

Previously, by means of tests on normal subjects, it was ascertained that 2-mercaptoimidazole was a potent antithyroid agent, which was effective in inhibiting iodine accumulation by the thyroid for twenty-four hours or longer in doses of 25 milligrams. Confirmation of its efficacy in the treatment of thyrotoxicosis was obtained by the procedure described in this communication. Twenty patients have been treated with this material in dosage of 20 to 100 milligrams per day. Even in severely thyrotoxic subjects a dose of 20 milligrams sometimes produced nearly complete inhibition of hormone synthesis lasting as long as eleven hours. In other patients a dose of 20 milligrams was only partially effective. In one patient, myxedema occurred in $4\frac{1}{2}$ months with a dosage of 40 milligrams per day, and in another in three months with 60 milligrams per day.

For purpose of diagnosis, the method described in this communication seems to be superior to that based on total iodine accumulation

when the diagnosis of hyperthyroidism is under consideration. There is a greater difference between persons with normal thyroid glands and hyperthyroid patients in this test; it is probably much less sensitive to differences in the prior iodine intake than is the other method, and it has the additional advantage that it can be performed after antithyroid therapy has been started. Its major disadvantage is that it does not distinguish between hyperthyroidism and hyperplastic goiter from some other cause.

When the question of myxedema is being studied and in investigating certain types of goiter, measurement of the rate of total iodine uptake without previous administration of an antithyroid compound is preferable.

SUMMARY

After virtually complete inhibition of thyroid hormone synthesis by antithyroid medication, the human thyroid gland was still able to collect appreciable quantities of radioactive iodine believed to be in the form of iodide ion. After administration of thiocyanate or a large dose of ordinary iodide, this collected radioactive iodide was promptly, but usually not completely, discharged from the thyroid gland. This phenomenon of loss of radioactive iodine from the thyroid by dilution with large quantities of ordinary iodide adds to the evidence from animal experiments that the radioactive iodine collected under conditions of previous thyroid inhibition is in the form of iodide ion.

The pattern of accumulation of I^{131} in thyrotoxic patients was distinctive in that the extent of uptake was much larger than in normal individuals, and the rate of discharge was rapid after thiocyanate or ordinary iodide administration. These differences are great enough to provide an accurate means of detecting active thyroid hyperplasia, which is most commonly manifested as thyrotoxicosis.

The extent of loss of the collected radio iodide occasioned by a standard dose of thiocyanate was related to the degree of previous inhibition of hormone formation. Thus, the adequacy of a schedule of antithyroid therapy can be determined shortly after beginning treatment, and without interrupting the medication. The duration of action of single doses of an antithyroid compound is readily ascertainable.

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SPIRAL ARTERIES IN THE HUMAN OVARY

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OCCASIONALLY it happens that a scientific observation is made only to be forgotten and rediscovered after a long period of time. When it is rediscovered, it may be remembered because its significance is appreciated in the light of newly acquired knowledge. The original discovery in 1774 of the spiral arteries in the human endometrium by William Hunter is a case in point. It was not until Daron rediscovered these structures in the uterus of Macacus rhesus in 1936 that their relation to physiological events of the menstrual cycle began to be appreciated.

The recent observation that the arterial supply in the rabbit ovary is derived from a helical vessel of diminishing diameter lying along the hilus of the ovary (Reynolds, 1947a) is a second case in point. The recent literature on the subject of ovarian morphology and physiology contains no generally known reference to the existence and possible function of this structure until 1947 when it became the subject of several studies (Reynolds, 1947). This structure has been shown to play a special role in adaptation of the arterial supply to changes in size of the ovary, and in regulation of blood pressure within, and blood flow throughout, the ovary.

When these studies were under way, a chance observation was made of a publication by Belou of Buenos Aires in 1934, in which spiral arteries were described in a single instance in the human ovary. Belou described these by saying, "La ovarica (referring to ovarian blood vessel) permite detaller los pelotones de vasos helicinos del hilo del ovario." This observation has remained unnoted apparently, without attracting further attention of physiologists, anatomists or clinicians.

After studies of the nature of the spiral artery in the human ovary were begun at Cumberland Hospital, it was found, again by chance, that a description of the arterial arrangement in the human ovary was given by Farre in 1858. The details of this description fit so exactly the specimens shown below that it may be quoted verbatim. It

reads, "The ovary derives its supply of blood chiefly from the ovarian (spermatic), but in part also from the uterine arteries. So free, indeed, is the communication between these vessels, that the organ may be equally well injected from either source. The communication is effected chiefly by means of a branch of the ovarian artery, which passes inwards to inosculate with a terminal branch of the uterine artery, this anastomotic branch being occasionally so large as to constitute the principal source of supply of the ovary. The terminal vessels are conducted to the lower border of the ovary between the

FIG. 1. Left ovary—Surgical—a 39 year old white woman, eighth pregnancy, four children—subtotal hysterectomy and bilateral salpingo oophorectomy. Ovarian artery injected. Stereoscopic view (×2). The vessels at the right have been moved out of their natural position.

folds of the posterior duplicature of the broad ligament, where they lie in parallel lines, and are readily distinguished by their tortuous or spiral form. Having entered the base of the organ, they spread out into those numerous ramifications which penetrate every part of the ovarian stroma, and give to this structure its peculiar fibrous aspect. From their extreme branches the blood is returned by the veins, which pass to the base of the organ, where they are very numerous. They form near the ovary and between the folds of the broad ligament, a plexus termed the ovarian or pampiniform plexus, the vessels of which communicate also with the uterine plexus. Valves are found in the ovarian veins only in exceptional cases."

Plastic casts of these vessels in three ovaries which were obtained by us are shown in Figures 1, 2 and 3.

This arrangement of the vessels in the human ovary was demonstrated in the manner previously described (3) as follows: The plastic, vinylacetate was injected into the vascular system of human ovaries. Surgical and post-mortem specimens were used. In some cases, in-

jection was made through the ovarian artery, and in others the uterine arteries were used. Equally good preparations were obtained in both instances, because of the free inosculation between the uterine and ovarian (spermatic) arteries in the hilus of the ovary to which Farre refers. The following procedure was used for injections:

FIG. 2. Left ovary—Post-mortem—a 39 year old white woman, twice pregnant with two living children—history of convulsions, hypertension and kidney disease. Uterine artery injected. Stereoscopic ($\times 1\frac{1}{2}$).

FIG. 3. Veins in the hilus of the ovary, showing the pampiniform plexus. Left ovary—post-mortem—a 53 year old white woman who was nephritic and diabetic. Ovarian vein injected. Stereoscopic ($\times 2$).

An 18 gauge hypodermic needle with blunted tip was inserted into the vessel to be injected. The tissue around it was tightly tied with a black silk ligature. The other arterial stump (either uterine or ovarian) was clamped. A small amount of acetone was injected and the cup of the needle filled. This precaution was taken to prevent premature

hardening of the plastic. Colored vinylite solution was then injected as rapidly and in as large quantity as possible. After fifteen minutes, the injected ovary was trimmed away from surrounding tissue and placed in the corrosion solution (HCl and pepsin) and incubated at 37°C. Depending upon the amount of sclerosis in the ovary, the resultant casts were digested free in ten to fifteen days. (The ovaries of a fetus required only twenty-four hours, as in the case of the rabbit's ovary.) Small, loosely adherent bits of tissue could be washed away under a gentle stream of tap water or by careful rotation in a beaker of water. Thus far, forty-five ovaries have been prepared in this way. Detailed studies of these in relation to the condition of the ovary at the time of injection will be reported elsewhere.

Inasmuch as we have acquired much knowledge concerning the physiology of the ovary in recent years, we may be expected to utilize this in studying the preparations obtained by this method. In this way, it may be possible to gain an insight into the etiology of certain pathological states of the ovary.

In the rabbit, for example, it has been found that the growth of the ovary resulting from gonadotropic stimulation causes orderly extension of the coils of the spiral artery in the ovary. In the presence of *corpus* hemorrhagicum cysts, however, there is distortion of the spiral artery during the period of follicular enlargement.

Preliminary study of the human preparations already available show marked differences in the vascularity of corpora lutea and of follicular cysts. Likewise, there are striking alterations in sclerotic ovaries, and the cast made from ovaries of a premature infant (7 month's gestation age) show a fascinating vascular tree upon which the complex spiral structures are destined to grow.

SUMMARY

Spiral arteries have been observed in the hilus of the human ovaries. These were demonstrated by means of plastic injection-corrosion preparations. These confirm in every detail the original and forgotten description of these structures by Farre in 1858.

Preliminary observations suggest a relationship between distortion of the spiral blood vessels and certain types of ovarian pathology.

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THE ROLE OF THE STEROID HORMONES IN THE RELAXATION OF THE SYMPHYSIS PUBIS OF THE GUINEA PIG[1]

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SINCE the discovery by Hisaw (1926) that the separation of the pubic bones in the guinea pig was under the control of a specific substance which was later called relaxin, several controversies have arisen. These controversies are concerned with the identity of this relaxative substance as distinct from the crystalline steroid hormones and with the role played by the steroids in the phenomenon of pubic relaxation.

Relaxin was first obtained from the blood of pregnant rabbits (Hisaw, 1926; Brouha and Simmonet, 1928; Tapfer and Haslhofer, 1935; Abramson, Hurwitt and Lesnick, 1937; Marder and Money, 1944). It has also been found in the blood of pregnant sows, dogs, cats, mares and in placentae of rabbits (Hisaw 1927 and 1929), blood of pregnant guinea pigs Hisaw (1927), Zarrow (1947), blood of pregnant women (Pommerenke, 1934; Abramson *et al.*, 1937), and corpora lutea of the sow (Hisaw, 1927; Brouha and Simmonet, 1928; Abramson *et al.*, 1937). Fevold *et al.* (1930a; 1930b; 1930c; 1932) prepared an active extract from the corpora lutea of sows containing 30 G.P. (guinea pig) units per mgm. of dry powder. This work was confirmed by Abramowitz *et al.* (1944) and extended by Albert, Money and Zarrow (1947) who were able to obtain an extract from the entire ovary of pregnant sows containing up to 500 G.P. units per mgm. of powder.

In 1931 de Fremery, Kober and Tausk secured pelvic separation with estrogen which was confirmed by Courrier (1931), Tapfer and Haslhofer (1935) and Dessau (1935). Furthermore, relaxation was reported after treatment with estradiol and progesterone (Fels, 1931; Möhle, 1933; Tapfer and Haslhofer, 1935; Haterius and Fugo, 1939). These observations led many investigators to question the validity of the relaxative substance as a distinct entity although Brouha and Desclin (1934) confirmed the original observation of Hisaw (1926) that relaxation in the estrinized guinea pig required only 6 to 8 hours

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after relaxin was administered whereas estrin alone required a number of days.

Hisaw *et al.* (1942 and 1944) confirmed the previous reports that progesterone caused pelvic relaxation, and pointed out that whereas relaxin produced its effect in 6 hours, progesterone required 72 to 96 hours. It was further indicated that the response to relaxin was identical in the castrated and castrated, hysterectomized guinea pig while progesterone was effective only in the castrated animal. In addition, it was shown that injection of estradiol and progesterone into rabbits with an intact uterus caused the formation of relaxin. In contrast to these experiments Fugo (1943) reported relaxation in castrated, hysterectomized guinea pigs after large doses of estradiol dipropionate and progesterone. Finally Courrier (1945) reported relaxation with (a) estrogen, (b) progesterone, (c) estradiol followed by progesterone, and (d) estradiol and progesterone given simultaneously. He obtained best results with both hormones given simultaneously but concluded that estrogen was the most important agent in relaxation and that it was unnecessary to postulate a new hormone.

Thus it is apparent that the situation with regard to the agents responsible for pelvic relaxation is most contradictory. The present investigation is an attempt to resolve the question of the number of substances responsible for separation of the pubic bones of the guinea pig and to determine whether pubic relaxation induced by the sex steroids is distinct from that induced by relaxin. It was also hoped that this study might shed additional light on the role of the uterus in the phenomenon of relaxation of the symphysis pubis.

MATERIAL AND METHODS

All guinea pigs were operated on at approximately 250 grams body weight and permitted to rest until they attained a body weight of 450 to 500 grams. The ovaries were removed under ether anesthesia by a lateral approach. The hysterectomies were performed by a similar lateral approach through which the ovaries and the upper part of the uterine horns were freed. This was followed by an abdominal incision through which the lower end of the uterus was freed, tied off posterior to the utero-cervical junction and removed. Thus in the castrated, hysterectomized guinea pig the ovaries, fallopian tubes, uterus and part of the vagina were removed.

The symphyses of the guinea pigs receiving injections of steroid hormones were palpated daily and recorded as negative, questionable, or positive, and in evaluating the data all questionable responses were regarded as negative. This is in accordance with the procedure followed in the routine assays for relaxin. At the conclusion of the treatment some of the animals were placed in metabolism cages and a 24 hour sample of urine collected under toluene for relaxin assay. Blood was obtained from all the animals by cardiac puncture and uteri removed, some of which were fixed in Bouin's for histological examination and others extracted for relaxin determinations.

Assays for relaxin content of urine, blood and tissues were carried out

with slight modifications according to the procedure of Abramowitz *et al.* (1944). The guinea pigs were primed with 1 μg of estradiol daily for three days and the extract to be tested was injected on the fourth day. The guinea pigs were palpated prior to the injection of the extract and only those animals with a tight pelvis were used. Six hours after injection of the extract the guinea pigs were palpated and results recorded as positive, negative or questionable. A guinea pig unit is defined as that amount of relaxin which produces definite relaxation of the symphysis pubis in two-thirds of a group of twelve or more castrated guinea pigs previously primed with 1 μg estradiol daily for three days.

Untreated blood serum was injected in a total volume of 1 ml. If a greater volume was required the serum was concentrated according to the procedure of Albert and Money (1946). When it was necessary to inject a small volume, the serum was always diluted to a constant volume of 1 ml. Urines were dialyzed in running water for 24 hours immediately after collection and when necessary concentrated by evaporation.

Tissue such as the ovary, uterus and vagina was ground up and extracted with 6 volumes of 3% HCl for 48 hours. The supernatant was removed by centrifugation and the residue re-extracted with 3 volumes of 3% HCl. The tissue was then discarded and the supernatants from the two centrifugations combined, neutralized to pH 7 to 7.4 and diluted for injection. If the volume was too great, it was reduced by precipitating with 5 volumes of acetone and redissolving the precipitate in saline at the desired volume. When it was necessary to store the extracts prior to carrying out the assays, the solutions were kept in the cold at an acid pH, under which conditions relaxin is stable.

The relaxin used in these experiments was prepared from whole, fresh sow ovaries (Albert, Money and Zarrow, 1947) and stored as a dry powder. Solutions of relaxin in normal saline were made up fresh as required. The steroid hormones,[3] estradiol, progesterone and desoxycorticosterone acetate were dissolved in sesame oil.

<div align="center">RESULTS</div>

Estradiol and Progesterone

A comparison was made of the effect of estradiol and progesterone on the time required for relaxation of the symphysis pubis of castrated and castrated, hysterectomized guinea pigs. Both groups of animals received 0.5 mgm. of estradiol daily for the duration of the experiment. Starting on the eighth day of estradiol treatment the animals also received 1 mgm. of progesterone daily in addition to the estradiol.

The results obtained (Table 1) indicate that treatment with large doses of estradiol and progesterone produced relaxation of the pubic symphysis of both the castrated and the castrated, hysterectomized guinea pig without the addition of any exogenous relaxin. However, the average relaxation time for the castrated guinea pigs was 10.4 days

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[3] The author is indebted to Schering Corporation, Bloomfield, New Jersey, for the progesterone, estradiol and desoxycorticosterone used in this study.

(range 10 to 11 days), while that for the castrated, hysterectomized group was 16.6 days (range 16 to 18 days), which is a significantly longer period. This difference is even more apparent if one compares the time required for pubic relaxation following the addition of progesterone to the treatment. The former group required 2.4 days while the latter group required 8.6 days. Assays for relaxin content of the blood and urine at the time of relaxation of the castrated guinea pigs showed 1 G.P. unit per ml. of blood serum and 1 G.P. unit per ml. of urine. The blood serum of the castrated, hysterectomized group

TABLE 1. TIME REQUIRED FOR PUBIC RELAXATION AND RELAXIN CONTENT OF BLOOD AND URINE OF CASTRATED AND CASTRATED, HYSTERECTOMIZED GUINEA PIGS AFTER TREATMENT WITH LARGE DOSES OF ESTRADIOL AND PROGESTERONE

G.P. no.	Treatment—daily		Time for relaxation		Relaxin content G.P. uints/ml.	
	Estradiol mgm.	Progesterone mgm. from 8th day	Total	Days following progesterone treatment	Blood serum	Urine
Group 1. Castrated, Hysterectomized						
1	0.5	1.0	16	8		
2	0.5	1.0	18	10		
3	0.5	1.0	16	8		
4	0.5	1.0	16	8		
5	0.5	1.0	17	9		
		Average	16.6	8.6	Neg. at 4 ml.	Neg. at 2 ml.
Group 11, Castrated						
6	0.5	1.0	11	3		
7	0.5	1.0	10	2		
8	0.5	1.0	10	2		
9	0.5	1.0	11	3		
10	0.5	1.0	10	2		
		Average	10.4	2.4	1	1

also tested for relaxin content at the time of relaxation, was negative when tested at a level of 4 ml. of serum and urine was negative at a 2 ml. level.

In a second set of experiments castrated and castrated, hysterectomized guinea pigs were treated with (a) 10 μg of estradiol and (b) 10 μg of estradiol plus 1 or 2 mgm. of progesterone. The estradiol was injected daily from the start of the experiment until the animals relaxed while the progesterone treatment was started on the 11th day of estradiol treatment and continued daily until the end of the experiment.

Castrated guinea pigs treated with 10 μg of estradiol alone relaxed in an average time of 23.7 days and castrated, hysterectomized guinea pigs given the same treatment required an average time of 25.6 days for relaxation, a difference that is not significant. However, castrated guinea pigs treated with 10 μg of estradiol and 1 mgm. of progesterone showed 90% relaxation with an average time of 13.5 days. With 2

mgm. of progesterone all the animals relaxed in the average time of 13.0 days. In contrast with this, castrated, hysterectomized guinea pigs treated with 10 μg of estradiol and 1 mgm. of progesterone required 23.7 days for pubic relaxation, a definitely significant increase in the time required when compared with the castrated group (Table 2). Furthermore, a comparison of these results based on the time required for pubic relaxation following the start of progesterone treatment indicated that the castrated animals required only 3.0 to 3.5 days whereas the castrated, hysterectomized animals required 13.7

TABLE 2. RELAXATION OF THE SYMPHYSIS PUBIS AND RELAXIN CONTENT OF BLOOD, URINE, AND UTERI OF CASTRATED AND CASTRATED, HYSTERECTOMIZED GUINEA PIGS AFTER TREATMENT WITH MODERATE DOSES OF ESTRADIOL AND PROGESTERONE

No. of G.P.	Treatment—daily		Average relaxation time days		Relaxin content		
	Estradiol μg	Progesterone mgm.	Total	Following progesterone treatment	Blood serum G.P. units per ml.	Urine G.P.U. per ml.	Uterus G.P.U. per gm.
Castrated							
9*	10	1 from day 11	13.5 (13–14)	3.5	0.5	0.3	10
10	10	2 from day 11	13.0 (12–14)	3	0.5	0.5	10
10	10	—	23.7 (16.31)	—	neg. at 4 ml.	neg. at 5 ml.	neg.
Castrated, Hysterectomized							
11	10	1 from day 11	23.7 (17–30)	13.7	neg. at 4 ml.	neg. at 8 ml.	—
10	10	—	25.6 (18–32)	—	neg. at 4 ml.	neg. at 4 ml.	—

* One guinea pig not included in the table required 22 days of treatment for pubic relaxation.

days. Finally it should be pointed out that the injection of 2 G.P. units of relaxin in either castrated or castrated, hysterectomized guinea pigs treated with estradiol for 10 days produced 100% pubic relaxation in 6 hours.

It is significant to note that relaxin was found in the blood, urine and uteri of castrated animals treated concurrently with both estradiol and progesterone whereas the castrated group given only estradiol and the castrated, hysterectomized animals given either estradiol or estradiol and progesterone showed no relaxin. The former group gave values of 0.5 G.P. units of relaxin per ml. of urine, and 10 G.P. units per gram equivalent of uterine tissue (Table 2).

Length of Progesterone Treatment

The treatment schedule used in the last experiment, wherein progesterone injections were started on the 11th day of estradiol treatment was an arbitrary one. Thus it was decided in the present study to determine whether starting the progesterone injections earlier than the 11th day of estradiol treatment would decrease the time required for pubic relaxation. A group of 5 castrated guinea pigs started with progesterone on the 6th day of estradiol treatment

showed a relaxation time of 13.2 days which is not significantly differ-
ent from the results obtained when progesterone injections were
started on the 11th day of estradiol treatment. Relaxin assays on the
blood and urine indicated 0.3 G.P. units per ml. of serum and 0.5
G.P. units per ml. of urine. Castrated, hysterectomized guinea pigs
treated in an identical fashion relaxed in the average time of 25.6 days
and the blood and urine were negative for relaxin (Table 3). Again
these results bring out in sharp contrast the difference in the time re-
quired for pubic relaxation between the castrated and the castrated,
hysterectomized guinea pigs.

A second group of 5 castrated guinea pigs received 10 μg of
estradiol and 2 mgm. of progesterone daily from the start of the ex-
periment. This group required approximately 8 days for relaxation of
the symphysis pubis, a significant decrease over the previous time
requirements. The blood serum of these animals showed 0.5 G.P.
units of relaxin per ml. (Table 3).

TABLE 3. THE EFFECT OF VARYING THE LENGTH OF TREATMENT OF PROGESTERONE ON
THE TIME REQUIRED FOR RELAXATION OF THE SYMPHYSIS PUBIS OF THE
CASTRATED AND CASTRATED, HYSTERECTOMIZED GUINEA PIG INJECTED
WITH BOTH ESTRADIOL AND PROGESTERONE

No. of G.P.	Treatment—daily		Average relaxation time—days		Relaxin content G.P. unit/ml.	
	Estradiol μg	Pro-gesterone mgm.	Total	Following progesterone treatment	Blood serum	Urine
Castrated						
5	10	2	(7–9)	8	0.5	—
5	10	2 from day 6	13.2 (11–14)	7.2	0.3	0.5
Castrated, Hysterectomized						
5	10	2 from day 6	25.6 (22–29)	19.6	neg.	neg.

Length of Estradiol Treatment

The purpose of this experiment was to secure data with regard to
the necessity of continuing the estradiol treatment after progesterone
injections were started in order to obtain pubic relaxation. A series of
castrated guinea pigs were injected with 10 μg of estradiol daily for a
total of 10 to 13 days and in all instances progesterone injections were
started on the 11th day.

When the castrated guinea pigs were given 10 μg of estradiol for
13 days and 1 mgm. of progesterone from the 11th day, 90% showed
pubic relaxation by the 14th day, i.e., 3 days following progesterone
treatment. However, when the estradiol was stopped on the 10th
day, only 10% of the guinea pigs relaxed (Table 4).

A more thorough investigation was made using a similar estradiol
injection schedule plus 2 mgm. of progesterone daily from the 11th
day. Estradiol treatment for 13 or 14 days resulted in pubic relaxation

TABLE 4. THE PER CENT OF CASTRATED GUINEA PIGS SHOWING PUBIC RELAXATION
AFTER TREATMENT WITH PROGESTERONE AND VARYING THE
LENGTH OF TREATMENT WITH ESTRADIOL

| No. of G.P. | Treatment—Daily | | % of G.P. Relaxed |
	Estradiol 10 μg daily for	Progesterone	
10	10 days	1 mgm. from day 11	10
10	13 days	1 mgm. from day 11	90
5	10 days	2 mgm. from day 11	0
5	11 days	2 mgm. from day 11	20
5	12 days	2 mgm. from day 11	80
10	13 days	2 mgm. from day 11	100
5	14 days	2 mgm. from day 11	100

in 100% of the animals within 14 days (2 to 3 days after the start of progesterone treatment). However, estradiol treatment for only 12 days plus a progesterone treatment similar to the above gave 80% relaxation by the 14th day. With a further decrease in length of estradiol treatment to 11 days only 20% of the animals relaxed, and finally when estradiol treatment was stopped on the 10th day and progesterone injections started on the 11th day (Fig. 1), no relaxation was obtained. Examination of blood and urine of the relaxed guinea pigs showed approximately 0.5 G.P. units of relaxin per ml. of blood serum and urine.
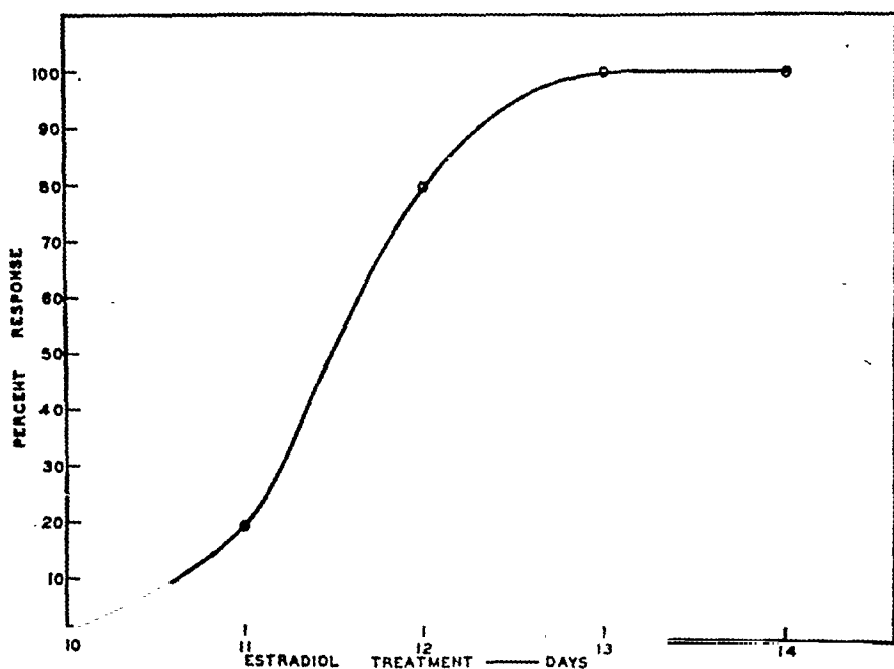

FIG. 1. Per cent of guinea pigs showing relaxation of the symphysis pubis, after treatment with estradiol and progesterone, plotted against length of estradiol treatment in days. The animals received 10 μg of estradiol daily and 2 mgm. of progesterone daily starting on the 11th day of estradiol treatment.

Estradiol and Desoxycorticosterone Acetate

The ability of desoxycorticosterone acetate and progesterone to act interchangeably under certain conditions made it seem worth while to investigate the effect of the former on the symphysis pubis of the guinea pig.

A group of 10 castrated guinea pigs were given 10 μg of estradiol daily for the duration of the experiment followed by 10 mgm. of desoxycorticosterone acetate daily from the 11th day of estradiol treatment. Relaxation of the pubic symphysis was obtained in 80%

TABLE 5. THE EFFECT OF DESOXYCORTICOSTERONE ACETATE ON RELAXATION OF THE SYMPHYSIS PUBIS OF GUINEA PIGS, TREATED WITH 10 μg OF ESTRADIOL DAILY THROUGHOUT THE EXPERIMENT AND WITH 10 MGM. OF DESOXYCORTICOSTERONE ACETATE DAILY FROM THE 11TH DAY OF ESTRADIOL INJECTIONS

G.P. No.	Time for relaxation days Total	days Following DCA treatment	Relaxin content G.P. units/ml. Blood serum	Urine	G.P. units/gm. Uterus
72	13	3			
73	did not relax				
74	13	3			
75	13	3			
76	14	4	0.25	—	10
123	13	3			
124	did not relax				
125	13	3			
126	13	3			
127	13	3	0.33	0.33	10
Average	13.1	3.1	0.29	0.33	10

of the animals with an average relaxation time of 13.1 days or 3.1 days after the start of DCA treatment (Table 5). Assays on the blood and uteri indicated 0.25 to 0.33 G.P. units of relaxin per ml. of serum, 0.33 G.P. units per ml. of urine and 10 G.P. units per gram of uterine tissue.

DISCUSSION

Different investigators have indicated that three separate substances may cause pubic relaxation in the guinea pig. These substances are relaxin (Hisaw, 1926 and 1929; Brouha and Simmonet, 1928; Brouha and Desclin, 1934; Abramson, Hurwitt, and Lesnick, 1937), estrogen (de Fremery, Kober, and Tausk, 1931; Courrier, 1931; Brouha, 1931; Dessau, 1935; Tapfer and Haslhofer, 1935) and a combination of estrogen and progesterone (Fels, 1931; Möhle, 1933; Tapfer and Haslhofer, 1935; Haterius and Fugo, 1939; Fugo, 1943; Hisaw, Zarrow, Money, Talmage, and Abramowitz, 1944). The fact that pubic relaxation may be produced by the sex steroids in addition to relaxin has not only tended to increase the confusion already present in this field, but has also led some investigators to doubt the existence of relaxin.

In 1934, Brouha and Desclin showed that relaxin produced pubic relaxation in a much shorter time than did the sex steroids. This was confirmed by Hisaw, et al. (1944) who indicated that while relaxin would produce pubic relaxation in 6 hours in the guinea pig, progesterone required 72 to 96 hours. Furthermore, relaxin was effective in both castrated and castrated, hysterectomized guinea pigs, whereas progesterone was effective only in castrated animals. In addition, these authors showed that progesterone caused the production of relaxin in rabbits with an intact uterus, and thus indicated that the necessity of an intact uterus for pubic relaxation by progesterone was additional evidence for the concept that progesterone and relaxin were separate entities. Fugo (1943) showed that massive doses of estradiol dipropionate plus progesterone caused pubic relaxation in the castrated, hysterectomized guinea pig and concluded the uterus was not essential for the relaxative effect of progesterone.

In the present investigation, the work of Fugo was repeated using the same injection schedule plus comparable amounts of estradiol and progesterone. It was found that the castrated, hysterectomized guinea pigs relaxed in the average time of 16.6 days which was similar to the results obtained by Fugo. However, the castrated animals relaxed in the average time of 10.4 days, a significantly shorter time. This difference is even more apparent when one compares the time required for pubic relaxation following the addition of progesterone to the treatment. The former group required 2.4 days while the latter group required 8.6 days. Furthermore relaxin assays of blood and urine were negative for the castrated, hysterectomized group, whereas the castrated animals showed approximately 1 G.P. unit per ml. of blood serum and 1 G.P. unit per ml. of urine. These results would seem to indicate that the uterus does play a significant role in the formation of relaxin, that relaxation produced by progesterone is mediated by the formation of relaxin in the body and differs from relaxation produced by estrogens. In view of the high doses of estradiol employed in the first experiment, the work was repeated with more physiological doses of estradiol and progesterone and a greater number of controls. It was found that castrated guinea pigs treated with estradiol and castrated, hysterectomized guinea pigs treated with estradiol alone or estradiol and progesterone relaxed in approximately the same time, namely in about 23 days. However, castrated animals treated with both estradiol and progesterone relaxed in approximately 13 days, a little more than half the time required by the other groups, and in addition relaxin was found in their blood, urine and uteri, whereas no relaxin was found in the castrated animals treated with estradiol or the castrated, hysterectomized animals treated with estradiol or estradiol and progesterone. Comparison of the time required for pubic relaxation following the addition of progesterone to the treatment, makes the difference in the time requirements more outstanding. The

castrated group required 3 to 3.5 days while the castrated, hysterectomized group required 13.7 days.

. Thus it is possible to produce relaxation of the symphysis pubis of the castrated, hysterectomized guinea pig by either estrogen or a combination of estrogen and progesterone. However, the time required for relaxation in the castrated, hysterectomized guinea pig is the same regardless of whether estrogen is given alone or in conjunction with progesterone. This would seem to indicate that in the guinea pig lacking a uterus, estrogen is the significant factor and the progesterone is not necessary.

However, in the castrated guinea pig with an intact uterus, treatment with progesterone in addition to estradiol, both shortens the time required for relaxation almost in half when compared with an estrogen treated group and also results in the appearance of relaxin in the blood, urine and uterus. Thus it seems that in an estrin primed, guinea pig with an intact uterus, progesterone causes the production of relaxin which results in the relaxation of the symphysis pubis.

It must also be pointed out that the present studies confirm the previous reports that the effect of injected relaxin on the symphysis pubis is noticeable in a matter of hours and is equally apparent in both castrated and castrated, hysterectomized animals. Injection of 2 G.P. units of relaxin in guinea pigs treated for 10 days with 10 μg of estradiol produced relaxation in 6 hours.

From these results one may conclude that relaxation of the symphysis pubis of the guinea pig may take place by means of two different procedures: (1) prolonged treatment with estradiol which apparently has a direct effect on the symphysis; (2) treatment with relaxin—exogenous relaxin may be injected into an estrogen primed guinea pig or endogenous relaxin may be formed in the animals with an intact uterus after treatment with estradiol and progesterone.

The concept that pelvic relaxation as induced by estrogen differs from that brought about by relaxin is strongly supported by the histological studies made by Talmage (1947). He found that the symphysis pubis when relaxed by giving estrogen showed resorption of bone and proliferation of loose fibrous connective tissue whereas relaxin produced a breaking up and dissolution of collagenous fibers. He also found that the histological changes in the symphysis in response to progesterone were identical with those following the injection of relaxin.

Previous work on relaxation by the sex steroids involved pretreatment with estrogens followed by injections of both estrogen and progesterone simultaneously. Consequently it was decided to test the need for the simultaneous injection of estrogen and progesterone after the pretreatment was finished. The results obtained indicate that estrogen for 10 days followed by progesterone will not cause pubic relaxation but a positive response occurs when estrogen is given for 13 days and progesterone is started on the 11th day. The important

difference between these two experiments is that in the latter instance there is an overlapping of treatment with both hormones for a period of three days. When there was an overlapping of two days, 80% of the animals responded and for one day only 20%. Thus it would appear that a certain amount of overlapping of the treatment with estrogen and progesterone is necessary for relaxation. This may also indicate that either relaxin was produced after a 10 day treatment with estradiol followed by progesterone but the guinea pigs did not relax due to a loss in the priming effect of the estradiol by the 13th day, or that simultaneous treatment with both hormones is needed for the production of relaxin in the body.

The ability of desoxycorticosterone acetate to produce pubic relaxation and the formation of relaxin in the blood of estrogen primed guinea pigs is not surprising. Previous investigators have shown that progesterone and desoxycorticosterone have similar activities such as the progestational effect on the uterus (van Heuverswyn, Collins, Williams, and Gardner, 1939; Hohlweg; 1939; Robson, 1939), growth of the mammary glands (Mixner and Turner, 1942), prolongation of life in adrenalectomized animals (Emery and Schwabe, 1936; Gaunt and Hays, 1938; Schwabe and Emery, 1939), protection against cold (Zarrow, 1942) and withdrawal bleeding in monkeys (Hisaw, 1935; Speert, 1940). The present results confirm the findings of Emery (1946) that desoxycorticosterone acetate like progesterone can cause pubic relaxation but disagree as to the ratio of activity. Emery found that desoxycorticosterone acetate and progesterone were about equally active, however, he had pretreated his animals with massive doses of estrogen. The present investigation indicates that the ratio of activity of progesterone to desoxycorticosterone acetate is approximately 1 to 10 in the production of pubic relaxation in the castrated guinea pig and that desoxycorticosterone acetate can also cause the formation of relaxin in the castrated animal.

SUMMARY AND CONCLUSION

Relaxation of the symphysis pubis of the guinea pig may be produced by treatment with (a) estradiol, (b) estradiol and progesterone and (c) estradiol and relaxin. In the castrated, hysterectomized guinea pig progesterone is without effect whereas in the presence of a uterus treatment with estradiol and progesterone both shortens the time required for pelvic separation as compared with estradiol treatment alone and results in the formation of endogenous relaxin. Relaxin is effective in both castrated and castrated, hysterectomized guinea pigs which have been pretreated with estradiol, and produces relaxation in six hours. This is a much shorter time than that following treatment with estradiol alone or estradiol and progesterone. It is concluded that relaxation of the symphysis pubis of the guinea pig may take place by means of two different procedures: (1) prolonged treatment with estradiol which apparently has a direct effect on the symphysis and (2)

treatment with relaxin—exogenous relaxin may be injected into an estrogen primed guinea pig or endogenous relaxin may be formed after treatment with estradiol and progesterone in the presence of a uterus.

It is noted that optimum relaxation with the steroids is obtained when progesterone and estradiol are given simultaneously. Desoxycorticosterone acetate is approximately one tenth as active as progesterone in its ability to produce the formation of relaxin and pubic relaxation.

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THE INFLUENCE OF MIXTURES OF ANDROSTERONE AND DEHYDROISOANDROSTERONE ON BIOLOGICAL RESPONSE

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We (Saier, Grauer and Starkey, 1943) and others (Friedgood and Whidden, 1941) observed that the chromogenic properties of androsterone were not altered by the presence of dehydroisoandrosterone in the same solution when varying mixtures of these two biologically active components were studied colorimetrically. As a result of this it was decided to observe the effect of mixtures of these androgens when determined by biological assay. Androsterone is known to be more active biologically than dehydroisoandrosterone. These normally occur in urine in a proportion of from $95\%+5\%$ to $85\%+15\%$. (Bauman and Metzger, 1940) (Callow and Callow, 1938). In pathological states, such as virilism caused by a tumor of the adrenal cortex, the amount of dehydroisoandrosterone may increase from the original 5 to 15% up to 40 to 70% of the excreted androgen (Callow and Callow, 1936). It is pertinent and at the same time appears paradoxical that as the less active fraction (dehydroisoandrosterone) is increased in amount the virilizing influence is enhanced. The question arose as to what effect this disproportion of the two hormones in solution will have on reactive tissue in the animal organism. In order to determine this the comb of the baby chick was selected as the target organ on which to observe the biological response of varying proportions of the two active androgens.

METHOD

In our preliminary studies were observed that the influence of light on the chicks' combs was an important factor in the weight response of this organ to the effect of androsterone. We found (Starkey, Grauer and Saier, 1940) that there were two variable factors that influenced the growth of the combs of the chicks; namely, the amount of androgenic material employed, and the intensity of the light to which the chicks were exposed. It therefore became evident that the light factor must be made constant and of adequate intensity. In order to achieve this the cages in which the chicks were kept

during the entire course of the experiment were wired with incandescent light bulbs of equal intensity. These cages were kept in a room that was thermostatically controlled for temperature and from which all extraneous light sources were eliminated by blackening of the windows. The lights were fastened at the tops of the cages so that they were all equidistant from the chicks. All of the chicks, including the control groups were exposed to this light source for 12 hours of each day. It was found that the visible light rays were the important factor in the spectrum since a group of chicks that were exposed only to ultra violet irradiation did not show the comb weight increment observed in those that were exposed to the visible light rays. In our present study we, therefore, applied this knowledge of the effect of light to the chick-comb-weight method of Dorfman and Greulich (1937).

Crystalline androsterone, dehydroisoandrosterone, and mixtures of varying proportions of these compounds[1] were dissolved separately in sesame oil and in 95% alcohol. We demonstrated (Starkey, Grauer and Saier, 1943) that an inert solvent such as sesame oil was preferable to alcohol as a vehicle for the routine biological assay of the androgens. In this study both of these vehicles were used in order to obviate the possible modifying influence of the solvent on the androgens in solution, and in order to act as a check on each other. The solutions and their proportionate mixtures that were employed were made up as follows:

> 100% Androsterone
> 95% Androsterone +   5% Dehydroisoandrosterone
> 75% Androsterone + 25% Dehydroisoandrosterone
> 60% Androsterone + 40% Dehydroisoandrosterone
> 50% Androsterone + 50% Dehydroisoandrosterone
> 40% Androsterone + 60% Dehydroisoandrosterone
> 25% Androsterone + 75% Dehydroisoandrosterone
>   100% Dehydroisoandrosterone

These proportions were chosen in order to establish the curve responses that could be expected in normal and in known pathologic states. The 95% androsterone +5% dehydroisoandrosterone mixture was taken to approximate the proportion present in normal urine. The mixtures that fell in the 25%+75% group and the 75%+25% group were taken to include the reported proportions that are found in adrenal cortical carcinoma. The solutions were made up at the beginning of each 6 day "run of chicks" and were used throughout that period of treatment. Particular care was taken to prevent evaporation of the alcoholic solutions by keeping them tightly stoppered and under refrigeration when not in use.

Exclusive of extensive preliminary work, 3,501 two-day old straight-run (unsexed) White Leghorn chicks were employed in this study. Of this number 118 were controls. All of the chicks were obtained from the same hatchery and were maintained under standard conditions of light and temperature inasmuch as we had demonstrated that variations in the intensity of light influenced the size and weight of the chicks' combs (Starkey, Grauer and Saier, 1940). The daily dose of the oily solution contained graduated doses of from 1 to 40 gamma and that of the alcoholic vehicle from 1 to 30 gamma of

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[1] The androsterone and dehydroisoandrosterone were kindly supplied by Ciba Pharmaceutical Products through the courtesy of Dr. Ernst Oppenheimer.

the separate and combined androgens. Daily inunctions were made on the chicks' combs for six consecutive days in a constant amount of 0.01 cc. The chicks were anesthetized on the seventh day and their body weights were recorded. The combs were then removed and weighed. From the average weights of groups of 12 to 32 chicks on a given dose, the response was expressed as a comb weight/body weight ratio. From these data suitable (parabolic and log) curves were calculated by the method of least squares. Since the CW/BW ratio of the controls was fairly constant (0.30+0.02), they were not considered in calculating the response of the experimental animals.

RESULTS

The individual solutions of 100% of androsterone and of 100% dehydroisoandrosterone gave, as was expected, biological responses which lay at the two extremes of the mixtures. We found the biological activity of crystalline androsterone in oil to be somewhat greater than twice that of the dehydroisoandrosterone (Figures 1 and 2). The
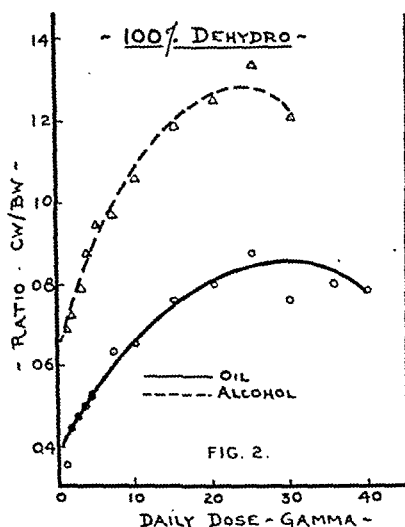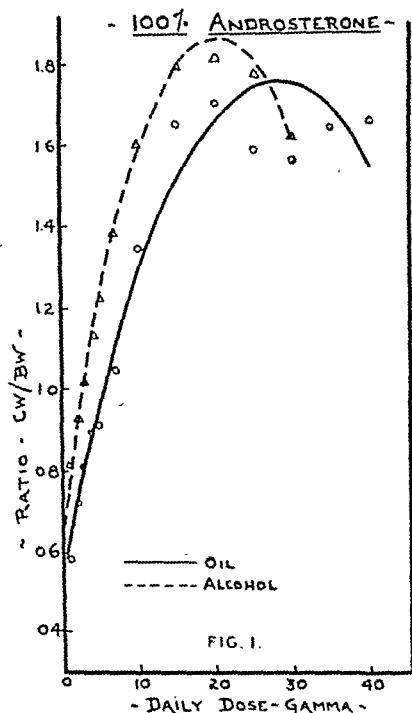
FIGS. 1 and 2. Curve responses of the individual solutions are established in oil and alcohol. The oily solution of androsterone is twice as active biologically as the dehydroisoandrosterone.

most significant findings lay between these two extremes. It is important to observe that the increase in the comb weights did not parallel the proportion of each androgen in the mixture. Thus, when 5% of dehydroisoandrosterone was mixed with 95% androsterone (Figure 7) the comb response approached that of 100% dehydroisoandrosterone

rather than that of androsterone. This surprising depressive action of only 5% dehydroisoandrosterone would lead one to expect the depressive biological effect of this androgen to increase in direct proportion to the amount present in the mixture. This, however, was not the case since the results that were obtained showed that the amount of the most biologically active substance did not control the order of response. Thus, when 25% of dehydroisoandrosterone was mixed with 75% of androsterone (Figure 3) the chicks' combs weights were slightly greater. This enhancement in biological response was further
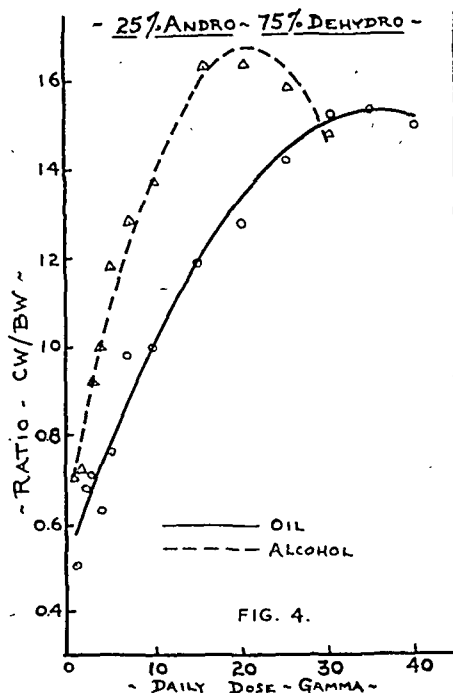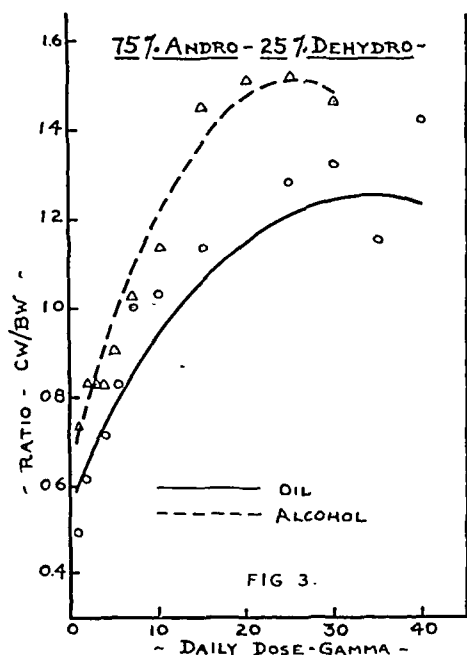
FIGS. 3 and 4. Mixture of 75% dehydroisoandrosterone (less active component) with 25% of androsterone gave the highest response in oily solution. Reversal of these proportions, gave a depressing response. As less active fraction was increased, tissue response increased.

observed as more dehydroisoandrosterone was added to the mixtures. Combinations of 60%+40% and of 40%+60% (Figures 5 and 6) showed practically no difference in response to each other, but did show an increment over the preceding mixtures in Figure 3. The mixture of 25% androsterone +75% dehydroisoandrosterone (Figure 4) gave further enhancement to the chick comb weight response. It was observed in all of our work, as evidenced by the curves, that the responsiveness of the chicks' comb to androgenic stimulation reaches a maximum point at a dose between 30 and 40 gamma, after which it declines. This was not the case when a mixture of equal parts of the two androgens were employed in an oily solution (Figure 8). At the 40 gamma dose, the chick comb weight response was still rising. This
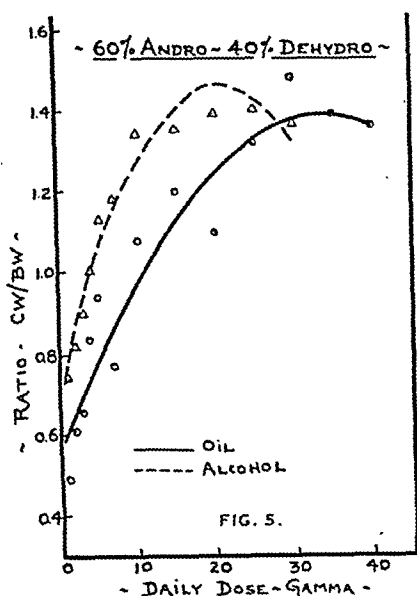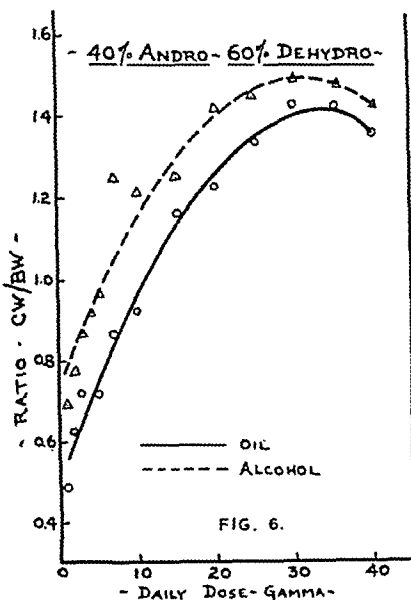
FIGS. 5 and 6. Mixtures of $60\%+40\%$ and $40\%+60\%$ were almost identical in response.
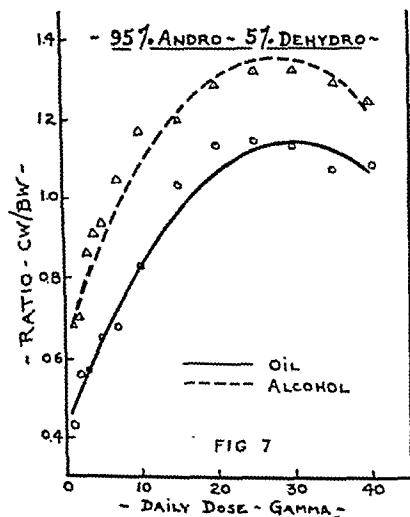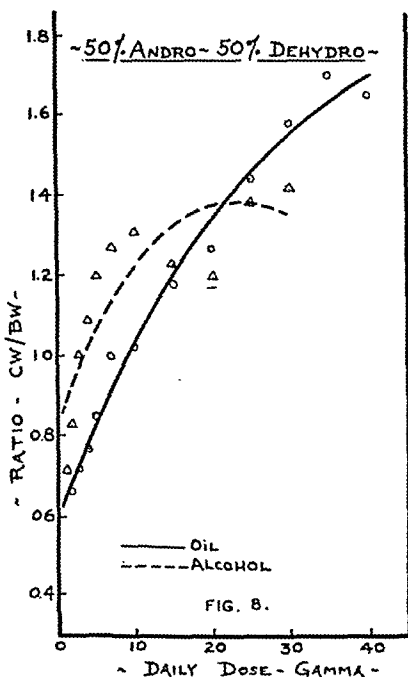
FIG. 7. A slight amount of the less active component (5% of dehydroisoandrosterone) depressed the biological activity of the androsterone.

FIG. 8. The oily solution of a 50–50 mixture gives a constantly rising response that can not be explained.

mixture was repeated on another "run" of chicks and was found to be correct.

The solutions of the androgens in alcohol gave greater enhancement of tissue response than those in oil, but we had observed that the oily solution was more consistently reliable. The order of response of the androgen mixtures in oil and in alcohol can be seen from the composite log curves plotted on semi-log graphs (Figures 9 and 10).
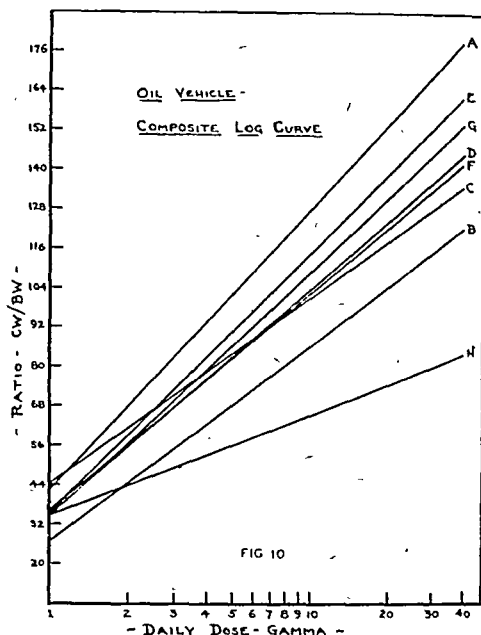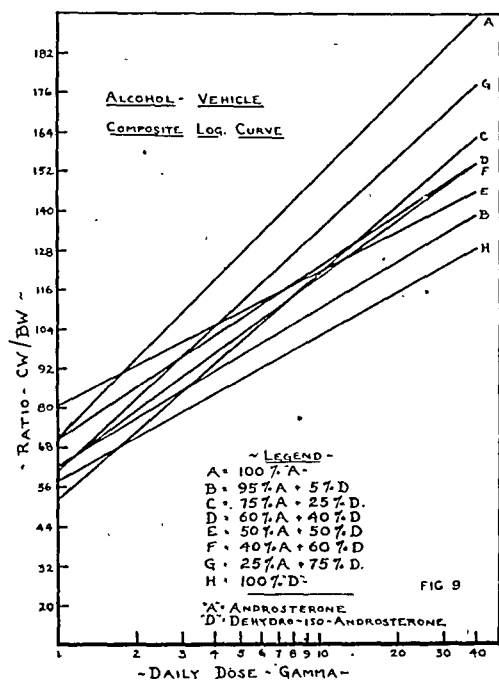
FIGS. 9 and 10. Composite log curves showing order of response of the varying mixtures.

In order to further determine the order of response of these mixtures and in order to attempt to evaluate the unusual response of the 50-50 mixture when an oily solvent was used another approach was taken. An arbitrary ratio of CW/BW (ratio 1.28) was chosen and the daily dose of the mixture that was required to reach that ratio was read from the log graph. The order of response was determined by the least amount of androsterone in the mixture of the daily dose that reached the given ratio (Table 1). From this chart, it may be seen that the biologically most active mixture of these androgens is 25% androsterone +75% dehydroisoandrosterone and that the least active is that of 95% androsterone +5% dehydroisaondrosterone. The other mixtures fall in progression so that as the dehyroisoandrosterone is increased, at the expense of the androsterone, the mixtures become increasingly more potent. The 50% androsterone +50% dehydroisoandrosterone

in oily solution is seen to be irregular due to its peculiar activity. The interpretation of this response is not clear.

DISCUSSION

We previously observed that mixtures of crystalline androsterone and dehydroisoandrosterone in the same solution did not affect the chromogenic properties of the components in the mixture. The colorimetric calibration curves for the mixtures of these two subtsances fell

TABLE 1. MIXTURES OF ANDROSTERONE AND DEHYDROISOANDROSTERONE
NECESSARY TO GIVE MAXIMUM BIOLOGICAL RESPONSE

Order of response determined by amt. of A	Alcohol vehicle			Oil vehicle		
	Mixtures of A & D*	Daily dose in gamma	Proportions of mixtures A D	Mixtures of A & D*	Daily dose in gamma	Proportions of mixtures A D
1	25 A–75 D	8.4	2.1 6.3	25 A–75 D	18	4.5 13.5
2	100 A	5.2	5.2 0	50 A–50 D	15	7.5 7.5
3	40 A–60 D	14	5.6 8.4	40 A–60 D	24.5	9.8 14.7
4	50 A–50 D	15	7.5 7.5	100 A	10	10.0 0
5	60 A–40 D	12.5	7.5 5.0	60 A–40 D	22	13.2 8.8
6	75 A–25 D	12.5	9.4 3.1	75 A–25 D	30	22.5 7.5
7	95 A– 5 D	24	22.8 1.2	95 A– 5 D	50	47.5 2.5
8	100 D	25.0	0 25.0	100 D—Reaches a ratio of only 0.84		

* A—Androsterone   D—Dehydroisoandrosterone.

Note: Table was determined by the daily dose that each mixture required in order to reach $\frac{\text{comb-weight}}{\text{body-weight}}$ ratio of 1.28. Order of response was determined by the least amount of androsterone in the mixture of the daily dose that would reach this ratio.

between those constructed for the individual hormones and were found to be equal to the sum of the components. Our studies on the chick's comb indicated that this mathematical response did not prevail with biological material, and that potentiation appeared to exist in certain proportionate mixtures between androsterone and dehydroisoandrosterone in vivo. Dehydroisoandrosterone, while much less active than androsterone when used alone, became very active when combined with a small amount of androsterone. However, a small amount of dehydroisoandrosterone combined with androsterone depressed the normally enhanced activity of the androsterone, resulting in a much depressed response. The important implications of these findings become immediately apparent when we review the altered proportion of these substances in the pathologic state in the human as compared to the excretions as found normally. Callow and Callow (1940) found that the relative proportions of androsterone, hydroxyetiocholanone, and dehydroisoandrosterone in normal male urine were 1.6, 1.4 and 0.2 mg. per liter, respectively. In the normal female the same steroids were present, androsterone and hydroxyetiocholanone being about equal and dehydroisoandrosterone being present in 10 to 15% of the total. In the eunuch the proportions were altered to 0.5, 0.9 and 2.0 mg. per liter. Conversely, in adrenal virilism in the female the dehydroisoandrosterone is increased up to 40 to 70% of the

total. Thus, while the total steroids may be the same in the urine from two individuals, the proportions of the biologically active androgens may vary greatly and be associated with abnormalities in the human.

Separation of the total 17-ketosteroids into the alpha and beta fractions (Talbot, Butler and MacLachlan, 1940) (Bauman and Metzger, 1940) would tend to indicate by colorimetric methods the proportions of androsterone and of dehydroisoandrosterone that occur in a given specimen of urine. Other components in the biological material being assayed may have a modifying influence that would not be indicated by the colorimetric values. Thus, the beta fraction will probably contain biologically active isoandrosterone as well as dehydroisoandrosterone. In the light of our observations, the biological influence of isoandrosterone on the beta fraction can be speculated upon but would not be revealed by colorimetric fractionation.

This raises another important consideration. Biological assays are generally interpreted from a standard androsterone response curve which is plotted from pure androsterone. When biological material, such as extracted urine, is employed the differing proportions of the two active androgens will give a response that is at variance with a fixed curve derived only from androsterone. Consequently, it appears advisable to employ curves that are determined from mixtures of the two active androgens in proportions approximating the normal human rather than from a curve derived only from androsterone.

With the almost universal use of the colorimetric method for 17-ketosteroid determination, a better understanding of the relations of chromogenic properties to biological activity is pertinent. We assayed numerous normal and pathological urine specimens colorimetrically, using the M-dinitrobenzene method (Zimmerman reaction) for total 17-ketosteroids and attempted to confirm the results biologically by the capon and chick-comb-weight method, employing aliquot portions of the same urine. A parallel trend was obtained but attempts to correlate the results obtained by the two methods were not successful. This failure was possibly due to a lack of knowledge of the proportions of the biologically active androgens contained in the "total androgen" fraction. Pincus (9) indicated that a number of neutral ketosteroids acted as chromogens in the Zimmerman reaction. These substances, particularly non-alcoholic ketones, tended to cause an overestimate of the 17-ketosteroids. By the use of antimony trichloride he was able to identify androsterone and its isomers spectrophotometrically and could differentiate them from other substances that were indistinguishable with the Zimmerman reaction. This report indicates that a close approximation of the biologically active proportions of an unknown can be determined colorimetrically. Until this is fully accomplished, our observations emphasize the fact that colorimetric determinations, as generally performed, may give misleading results if

attempts are made to interpret them in relation to their possible biologically active proportions.

SUMMARY

It was found that mixtures of varying proportions of androsterone and dehydroisoandrosterone gave evidence of potentiation when the chick-comb-weight method of measurement was employed. Though pure androsterone is much more active biologically than dehydroisoandrosterone, it was found that a small dose of dehydroisoandrosterone (5%) had a marked biologically depressing action on androsterone. Paradoxically, as the amount of the biologically less active component (dehydroisoandrosterone) was increased in the mixture the biological response of the chicks' comb was enhanced. Contrary to anticipation, a mixture of 5% dehydroisoandrosterone +95% androsterone gave the most depressing response of the chicks' comb, while 75% dehydroisoandrosterone +25% androsterone gave the greatest response. Apparently the amount of androsterone in the mixture does not control the order of response of the biological material. The correlation of 17-ketosteroids with biological urinary assays for androgens is thus interfered with unless there is knowledge of the proportions of the active androgens present. The response of chicks' combs to the varying proportions of mixtures of biological active androgens was controlled by a constant light source.

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NOTES AND COMMENTS

PLASMA AND LIVER PROTEIN CONCENTRATIONS OF HYPOTHYROID RATS[1]

THIOURACIL and thiourea adminstration is followed by a significant increase in liver weight (Leblond and Hoff, 1944, May, Moseley and Forbes, 1946, Leathem and Seeley, 1947). Neutral fat and cholesterol levels do not change after thiourea feeding whereas liver glycogen has been reported as markedly increased (May, Moseley and Forbes, 1946) as well as decreased (Glock, 1945). The percentage of water and protein in these enlarged livers is normal so that an increase in liver protein per unit of body weight results (Leathem and Seeley, 1947). Leblond and Hoff (1944) indicate that liver atrophy due to thyroidectomy is not observed in the thiouracil induced hypothyroid state suggesting a unique action of thiouracil. This becomes more apparent in view of the reduction in food intake which follows either attempt to creat hypothyroidism as this in itself will decrease liver size and protein (Kosterlizt and Cramb, 1943) (Harrison and Long, 1945). In this report the effect of thiouracil feeding on the liver is compared with thyroidectomy and in each instance non-treated controls were pair fed.

Total plasma protein, plasma globulin and non-protein nitrogen increase in concentration while plasma albumin remains unchanged in male rats fed 0.5% thiouracil for 20–25 days (Leathem and Seeley, 1947). Rats that have been thyroidectomized exhibit similar plasma changes in comparison with ad libitum fed controls (Levin and Leathem, 1942) (Moore, Levin and Smelser, 1945) and are here compared with rats on controlled food intake.

Male rats of the Long-Evans strain were used at 150 days of age and kept in metabolism cages for measurement of daily food intake. Purina fox chow (22.2% protein) was fed ad libitum to thyroidectomized rats as well as to those receiving thiouracil[2] as 0.5 per cent of the diet. The experimental period was 20–25 days in duration after which time the rats were bled by cardiac puncture under light ether anesthesia and the plasma analyzed for non-protein nitrogen, total protein, albumin and globulin concentrations by methods reported previously (Leathem, 1945). The liver was dried to constant weight and was then ground to uniform consistency and analyzed for total nitrogen. Nitrogen values were converted to protein by use of the factor 6.25.

Figures 1 and 2 illustrate the effect of 0.5% thiouracil feeding and thyroidectomy on food intake as compared with a general average of 16 gms. per day per rat eating ad libitum. It is readily apparent that a return to normal food consumption was a slightly slower process for the operated rats. In both groups a loss in body weight was recorded as a reflection of the subnormal

food intake, the pair fed controls showing body weight losses at least as great as the experimental animals.

Plasma protein concentrations revealed trends of similar nature in thiouracil fed and thyroidectomized rats. NPN, total plasma protein and
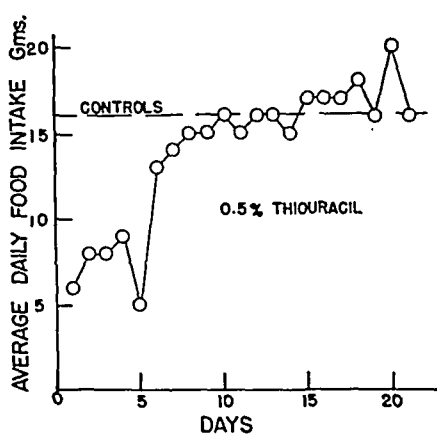
FIG. 1
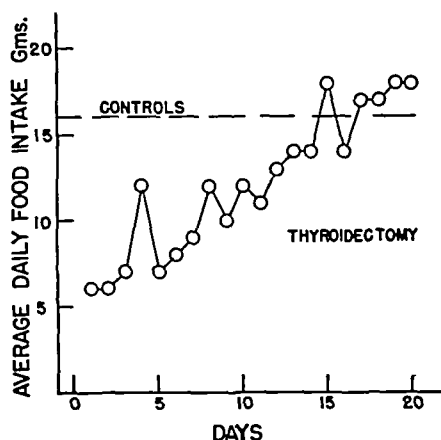
FIG. 2

plasma globulin concentrations increased while plasma albumin levels did not differ from those in pair fed controls. The hematocrit decreased in both hypothyroid states (Table 1).

TABLE 1. COMPARATIVE EFFECT OF THIOURACIL AND THYROIDECTOMY ON BODY WEIGHT AND PLASMA PROTEIN LEVELS OF RATS

No. of rats	Treatment	Body weight start end	Hemato-crit	Non-protein N	Total protein	Albumin	Globulin
		gm.	%	mg./100 cc.	gm./100 cc.	gm./100 cc.	gm./100 cc.
8	thiouracil (0.5%)	330–320	43.6±0.5*	59.7±1.0	6.68±0.21	3.39±0.17	3.29±0.17
8	controls	336–320	45.9±0.5	52.0±1.9	5.75±0.19	3.11±0.15	2.63±0.10
9	thyroidectomy	315–294	41.1±0.7	50.0±2.0	6.50±0.21	2.91±0.17	3.59±0.14
9	controls	315–282	46.3±0.9	45.0±0.8	6.11±0.20	2.96±0.09	3.15±0.11

$$* \epsilon = \sqrt{\frac{\Sigma d^2}{N(N-1)}}$$

The increase in liver size was again obtained with thiouracil and this liver tissue had a water and protein content comparable to those in control rats. Therefore, the total liver protein was significantly increased. Thyroidectomy, inducing a more rapid hypothyroidism than thiouracil, failed to increase liver size or to alter water or protein content (Table 2). Thus the effect of thiouracil on the liver would appear not be to due to hypothyroidism.

TABLE 2. COMPARATIVE EFFECT OF THIOURACIL AND THYROIDECTOMY ON THE LIVER OF RATS

No. of rats	Treatment	Weight gm. gm./100 gm. BW	Water %	Total protein gm.	Protein % dry wt.	Protein gm./100 gm. BW
8	thiouracil (0.5%)	15.2±0.6 4.2±0.2	71.0±0.3	2.93±0.13	66.9±1.2	0.886 (0.801–1.051)
8	controls	10.9±0.4 3.3±0.2	70.1±0.6	2.27±0.10	69.9±1.7	0.720 (0.631–0.790)
9	thyroidectomy	10.0±0.3 3.2±0.1	69.2±0.5	2.03±0.07	71.4±2.1	0.570 (0.610–0.825)
9	controls	9.5±0.5 3.4±0.2	70.3±0.2	2.02±0.06	74.0±2.4	0.710 (0.650–0.813)

SUMMARY

A rise in NPN, plasma globulin concentration and in total plasma pro-
tein occured in male rats 20 to 25 days after thyroidectomy or thiouracil feed-
ing. Plasma albumin concentrations simulated those of pair fed controls.
The hematocrit decreased.

Thiouracil induced an increase in liver weight while maintaining normal
water and protein percentages. Total liver protein was increased. These
liver effects were not duplicated in thyroidectomized rats.

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THE EFFECT OF DIETARY PROTEIN CONTENT UPON THE NITROGEN RETENTION AND WEIGHT GAIN PRODUCED BY THE HYPOPHYSEAL GROWTH HORMONE

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INTRODUCTION

THE FACT that hypophyseal extracts rich in growth hormone produce nitrogen retention has been shown repeatedly by many workers (Teel and Watkins, 1929; Teel and Cushing, 1930; Gaebler, 1933; Lee and Shaffer, 1934; Shaffer and Lee, 1935; Marx *et al.*, 1942; and Young, 1945). Purified growth hormone has the same effect in alloxan-diabetic rats (Bennett and Li, 1947), and in rats with increased nitrogen excretion following bilateral femur fracture (Bennett, Applegarth, and Li, 1946). Since the nitrogen retained must be derived from the dietary intake and be incorporated into normal body tissues, it would be reasonable to expect that the nitrogen retaining effect of a given amount of growth hormone would be influenced by the biological quality and the quantity of protein ingested. In these experiments we have used a standard dose of the growth hormone and have compared the amounts of weight gained and of nitrogen retained by rats fed constant amounts of iso-caloric diets with varying protein content.

METHODS

"Plateaued" female rats of the Long-Evans strain aged seven to ten months were used. The animals were fed purified diets in which alcohol-

washed casein was the protein source and which contained optimal amounts of all known dietary factors. All diets contained 8 per cent fat, 4 per cent salts (Hegsted et al., 1941), and 0.5 per cent liver fraction powder. The casein content of the five diets was 6 per cent, 12 per cent, 18 per cent, 24 per cent, and 48 per cent respectively, and the remainder of each diet consisted of sucrose, so that all diets were iso-caloric.

To each Kg. of diet were added thiamine chloride 5 mg., pyridoxine hydrochloride 5 mg., riboflavin 10 mg., p-aminobenzoic acid 10 mg., nicotinic acid 20 mg., calcium pantothenate 50 mg., inositol 500 mg., choline chloride 1 gm., and 2 methyl 1,4 naphthoquinone 5 mg. Each week each animal was fed the following fat soluble vitamins in 650 mg. of corn oil (Mazola): 6 mg. of alpha-tocopherol, 115 chick units of Vitamin D, 800 U.S.P. units of Vitamin A, one-seventh of the weekly amount being added to the daily feeding. The 24 per cent casein diet without liver fraction powder has been shown to support growth equal or superior to that produced on optimal stock diets of natural foods (Nelson and Evans, 1947). The "liver fraction powder" (Lederle 7-5293) has subsequently been added to supply "unknown Vitamin B_2 factors" and to insure completeness of the diet according to current concepts of nutrition.

It was found that 12 gm. of diet, weighed to 0.1 gm., was the maximum amount that each animal could be relied upon to eat per day, and this amount of diet was fed daily to each animal in each experiment. It should be emphasized that in all experiments all animals received the same caloric intake, no attempt being made to compensate for the varying specific dynamic actions of the diets. No rat was used in more than one experiment. The animals were weighed at the same time daily, immediately before being fed.

After an adaptation period of from three to seven weeks on the purified diets, the rats were placed in individual screen-bottomed cages. Urines were collected through acid-moistened, ribbed glass funnels into wide-mouth Erlenmeyer flasks containing approximately 10 cc. of 1N H_2SO_4, feces and hair being separated from the urine upon perforated porcelain discs. Funnels were washed with distilled water and urines collected at 48-hour intervals except during injection periods when urines were collected at 24-hour intervals. Urines were analyzed for nitrogen content by the micro-Kjeldahl method and are reported as 24-hour nitrogen excretion.

At each dietary level the weights and urinary nitrogen excretions of each of ten rats were determined for ten days prior to injection. Injection periods of five days were used during which half of the animals received 0.5 mg. of growth hormone intraperitoneally twice daily while the others were similarly injected with an equal amount of serum albumen. The hormone preparation used was prepared according to the previously published method (Li, Evans, and Simpson, 1945). Following the period of injections, the animals were similarly studied for a second control period of ten days duration.

EXPERIMENTAL DATA

The effect of the hypophyseal growth hormone upon the body weight and urinary nitrogen excretion of rats fed the 6 per cent casein diet is reported elsewhere (Gordan, Bennett, Li and Evans, 1947). It was shown that on the 6 per cent casein diet growth hormone produced

nitrogen retention without significant gain in weight. When methionine was added to the diet, growth hormone produced nitrogen retention and concomitant weight gain. With the methionine supplement five control animals showed an average weight increase of 4 gm. (range 3 to 7) in five days while five treated animals showed an average

Effects of Growth Hormone on Body Weight and
Nitrogen Excretion of Rats on 12 % Casein Diet
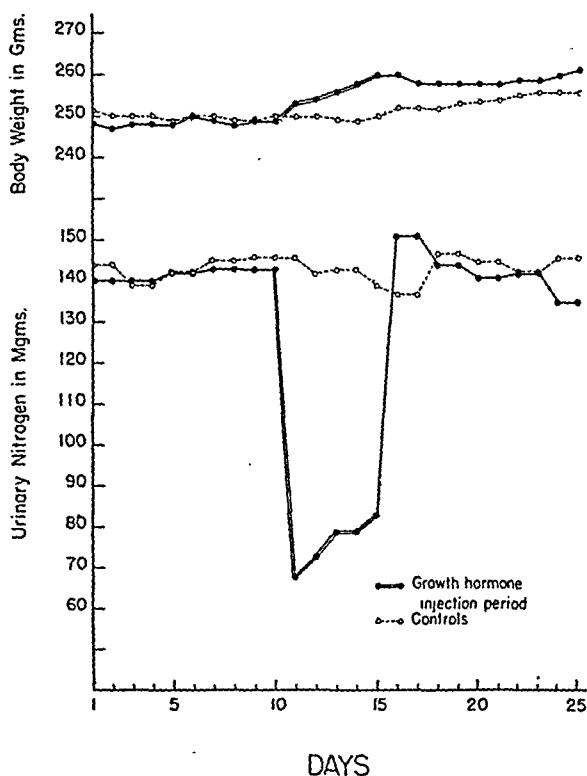
Fig. 1

weight gain of 13 mg. (range 10 to 15). The difference in the increment of weight between the control and growth hormone injected rats was calculated as a net gain of 9 gm. ascribed to the action of growth hormone. The control rats excreted an average of 87 mg. of nitrogen per day of injection while the growth hormone treated rats excreted only 48 mg. per animal per day. The difference was calculated as a net retention of 39 mg. of nitrogen per animal per day of injection.

With dietary casein contents of 12 per cent, 18 per cent, 24 per cent, and 48 per cent, no amino acid supplements were used. The data and their statistical analyses are presented in Table 1. A graphic picture of the day by day body weight changes and nitrogen excretion

TABLE 1. EFFECT OF GROWTH HORMONE ON URINARY NITROGEN EXCRETION AND BODY WEIGHT OF RATS

Diet	Group	Daily nitrogen excretion				Nitrogen retention produced by growth hormone†	Change in weight during treatment period	Increment in body weight due to growth hormone‡	Theoretical increase in body weight§
		Ten day preinjection period	Five day injection period		Ten day post-injection period				
		mgs.	mgs.	p*	mgs.	mgs.	gms.	gms.	
6% casein plus methionine	Controls	77 ± 2.3	87 ± 2.4	<0.01	92 ± 2.0	+39	+4	+9	6.1
	Treated	68 ± 2.3	48 ± 2.3		93 ± 3.9		+13		
12% casein	Controls	143 ± 2.9	143 ± 2.7	<0.01	146 ± 2.5	+67	0	+11	10.5
	Treated	142 ± 2.2	76 ± 2.0		143 ± 2.3		+11		
18% casein	Controls	205 ± 1.7	206 ± 2.3	<0.01	206 ± 1.6	+92	+3	+15	14.4
	Treated	206 ± 1.6	114 ± 2.0		209 ± 1.6		+18		
24% casein	Controls	369 ± 7.5	371 ± 9.6	<0.01	350 ± 14.5	+119	0	+21	18.6
	Treated	338 ± 7.7	252 ± 11.1		372 ± 8.9		+21		
48% casein	Controls	716 ± 22.2	687 ± 19.8	<0.01	647 ± 27.0	+121	+2	+18	18.8
	Treated	723 ± 14.3	566 ± 15.6		740 ± 23.6		+18		

* From Fisher's (1946) table of t comparing the significance of the mean excretion of the treated and control animals during the injection period.
† The difference between the excretion of the control and treated animals during the treatment period.
‡ The difference between the weight change of the treated and control animals.
§ Calculated as explained in the text.

is presented in Figures 1 to 4. By reference to Table 1 it will be noted that the average daily retention of nitrogen produced by the growth hormone was 67 mg., 92 mg., 119 mg., and 121 mg. for the groups fed 12 per cent, 18 per cent, 24 per cent, and 48 per cent casein diets re-

Effects of Growth Hormone on Body Weight and Nitrogen Excretion of Rats on 18 % Casein Diet

DAYS
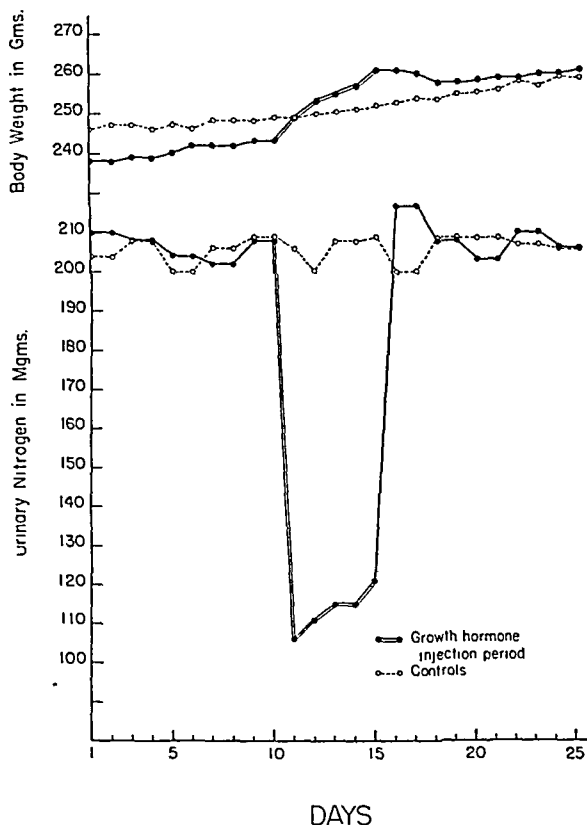
FIG. 2

spectively. The amount of weight gain likewise increased with the increasing dietary protein content until the 24 per cent casein level was reached.

DISCUSSION

It would appear from our data that there is a correlation between the amount of nitrogen retained and the amount of body weight gained during the five-day injection period. F. G. Young (1945) has shown by careful carcass analysis that the nitrogen retained, calculated as protein plus protein-bound water, accounts quantitatively for the amount of weight gained. Assuming that the tissue formed is 20 per cent protein and that the protein is 16 per cent nitrogen, a theo-

Effects of Growth Hormone on Body
Weight and Urinary Nitrogen Excretion of
Rats on a 24 % Casein Diet

DAYS
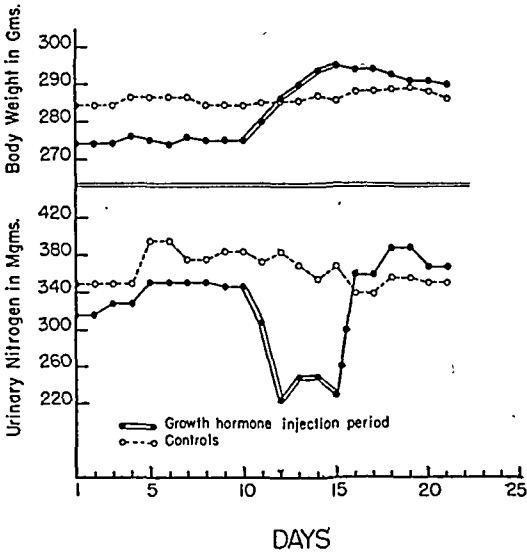
FIG. 3

Effects of Growth Hormone on Body
Weight and Nitrogen Excretion of Rats on
48 % Casein Diet

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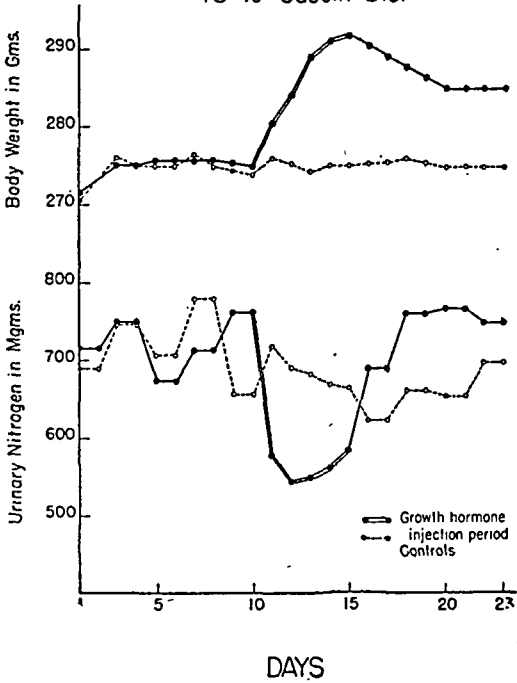
FIG. 4

retical weight increase can be calculated which corresponds strikingly with the amount of weight gain actually observed in the experiments (Table 1).

The relation of the body weight gains and the degree of nitrogen retention to the dietary protein content are graphed in Figure 5. It will be observed that the amount of nitrogen retention and increment in body weight produced by a standard dose of the growth hormone under these experimental conditions both increase in a linear fashion

Effect of Dietary Protein Level on Nitrogen
Retention and Weight Increase Produced by Growth Hormone
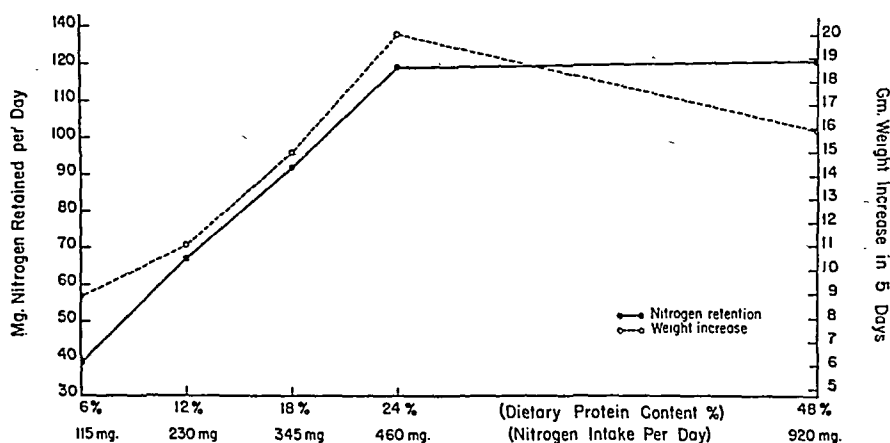
FIG. 5

from the 6 per cent to the 24 per cent casein diet and then fail to show any further increase when the dietary protein content is doubled.

These experiments have shown a characteristic pattern of response to the growth hormone which perhaps would be present only under these experimental conditions. Following the injection of growth hormone there is a prompt decrease in the urinary nitrogen excretion, usually maximal in the first 24 hours. Decreased urinary nitrogen excretion persists during the entire five-day injection period. Following cessation of injection the urinary nitrogen excretion promptly returns to the pre-injection level or is slightly higher.

Concomitant with the nitrogen retention is a proportionate increase in body weight. While the animals usually show the most rapid weight gain in the first 24 hours, they continue to gain weight throughout the injection period. Following cessation of injection the treated rats usually lose approximately 20 per cent to 30 per cent of the gained weight by the fourth post-injection day, then regain this in part or wholly to plateau once more on the tenth post-injection day at a level at or slightly below the maximal weight attained. The weight loss may be at least to some extent attributable to the excretion of some of the

previously retained nitrogen. Following cessation of injection the urinary nitrogen excretion returns to pre-injection levels or is slightly higher but never markedly so.

SUMMARY

The action of a standard daily dose of growth hormone for a five-day injection period on the body weight and urinary nitrogen excretion of rats fed complete diets is characteristically as follows: (a) the nitrogen excretion promptly decreases, usually to the greatest extent in the first 24 hours; (b) the body weight promptly increases; (c) upon cessation of injection, the nitrogen excretion promptly returns to the pre-injection level or is slightly higher; (d) upon cessation of injection approximately 20 per cent to 30 per cent of the weight gained is lost, the weight usually reaching the lowest level on the fourth post-injection day. The rats then regain weight to "plateau" again on the tenth post-injection day at or near the maximal weight attained.

The amount of nitrogen retained and the increment of weight gained under the influence of a five-day injection period of the hypophyseal growth hormone both vary concordantly with the dietary protein content. The 24 per cent casein diet is optimal for the dose of growth hormone which was employed.

The amount of nitrogen retained, calculated as protein plus protein-bound water, corresponds closely to the observed weight gain.

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CHEMICAL CHANGES IN THE BLOOD COMPO-
SITION OF CHICKENS AND TURKEYS FED
SYNTHETIC ESTROGENS

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PHYSIOLOGICAL changes in the lipid metabolism of the domestic fowl produced by the administration of estrogens have been referred to frequently in the literature (Entenman, Lorenz and Chaikoff 1938; 1940; Lorenz, Chaikoff and Entenman 1938). Lorenz and his co-workers, however, were the first to suggest a practical application of these facts in fattening market poultry through the use of estrogen pellet implants (Lorenz, 1943; 1944). Articles by Jaap and Thayer (1945) further increased the interest among poultrymen by calling attention to the possibility of fattening poultry by administering certain synthetic estrogens orally rather than by pellet implants as had been suggested by Lorenz. These articles also called attention to the fact that the physiological action of various synthetic estrogens was not necessarily similar for all poultry, since the order of estrogen activity was not parallel for chickens and turkeys.

In conducting fattening tests, a guide was needed to determine the amount of estrogen to be administered and the length of the fattening period. This need was best met by chemical analyses of blood and tissue of the experimental birds. During the past four years thousands of chemical determinations have been completed under the supervision of the authors of the present paper. Articles have been published summarizing some of the outstanding observations, but it is felt that more complete data should be on record. This paper reports the average blood analyses for fryers, broilers, cockerels, mature hens, cocks, and turkeys which had been fed fattening rations containing different levels of synthetic estrogens.

EXPERIMENTAL

Some work was done with market poultry in which solutions of estrogens were injected subcutaneously and estrogen pellet implants were made. Most of the results herein reported are from chickens and turkeys which received the estrogens dissolved in an oil vehicle and administered orally as an ingredient of a balanced basal fattening ration. Feeding was conducted under practical conditions, but with

the fowls confined in poultry houses where feed consumption data could be obtained. In some cases individual cages or fattening batteries were used. The chickens and turkeys were of various breeds selected from the Oklahoma Agricultural Experiment Station flocks.

Blood for analysis was drawn either by heart puncture or from wing veins in a hypodermic syringe and delivered into containers which had been previously coated by evaporating a solution of lithium citrate. In some cases samples were centrifuged to measure

TABLE 1. CHICKEN BLOOD ANALYSES
Average of 20 Pooled Samples from Mature Hens

Cells in whole blood, per cent	27.4
Plasma in whole blood, per cent	72.6
Glucose, mg./100 ml. blood	214.7
Non-protein nitrogen, mg./100 ml. blood	33.3
Urea, mg./100 ml. blood	5.7
Uric acid, mg./100 ml. blood	4.48
Creatinine, mg./100 ml. blood	2.13
Total phosphorus of whole blood, mg./100 ml.	122.3
Total phosphorus of cells, mg./100 ml.	284.2
Total phosphorus of plasma, mg./100 ml.	35.7
Inorganic phosphorus of plasma, mg./100 ml.	4.28
Calcium of plasma, mg./100 ml.	20.9
Free cholesterol of whole blood, mg./100 ml.	60.9
Total fatty acid of whole blood, mg./100 ml.	827.5
Carotene of plasma, micrograms/100 ml.	287.0
Vitamin A of plasma, micrograms/100 ml.	71.6

the cell-plasma ratio and to obtain a clear plasma for analysis. It was frequently observed that the blood from birds receiving certain synthetic estrogens had a light yellow color and a layer of fat appeared on top of the hematocrit tube. The plasma and blood were analyzed by the usual clinical methods (Schoenheimer and Sperry, 1934; Stoddard and Drury, 1929; Youngburg and Youngburg, 1930) at daily and weekly intervals during the fattening period and in some cases during a period of time after the estrogen feeding had been discontinued. Space permits reporting only the average results at certain intervals throughout the period, to show general trends.

Chickens

As a basis for later comparison, the average constituents of the blood from chickens consuming the basal ration under normal station conditions was first determined and is recorded in Table 1. The average analyses of blood of twenty or more separate pooled samples are recorded in all tables.

The next step was to find which blood constituents gave the best index of the effect of estrogens, since the number of analyses that could be made for separate compounds was limited by the necessity of following the effects of many types of synthetic estrogens on the blood of many hundreds of individual birds. Therefore rather complete

analyses were made of blood samples from 12-week-old fryers receiving the basal ration as a control and the basal ration supplemented with the indicated amount of dianisylhexane, dianisylhexene, dianisylhexene plus lecithin, and lecithin alone. An inspection of the results listed in Table 2 indicated that two constituents, namely free cholesterol and total fatty acids, made significant changes under

TABLE 2. BLOOD ANALYSES OF NORMAL AND ESTROGEN FED
TWELVE-WEEK-OLD FRYERS OF BOTH SEXES

Date	Feed	Whole blood total P	Plasma			Whole blood fatty acid	Cholesterol	
			Total P	In-organic P	Lipoid P		Whole blood free	Total
			(mg. per 100 ml. blood)					
4-23	Control	112	25	5.4	8.4	443	50	105
	40 mg. dianisylhexane/lb. ration	111	28	6.1	12.0	473	49	116
	50 mg. dianisylhexene/lb. ration	112	35	6.2	14.0	765	52	113
4-30	Control	116	15	2.4	9.8	325	42	100
	40 mg. dianisylhexane/lb. ration	139	83	4.2	44.0	2587	214	322
	50 mg. dianisylhexene/lb. ration	170	100	5.6	60.0	3622	305	324
5-6	Control	126	29	5.8	16.6	399	70	160
	40 mg. dianisylhexane/ration	174	135	14.5	39.6	1582	202	342
	50 mg. dianisylhexene/lb. ration	142*	155*	15.2*	60.0	2276	392	438
5-7	Control+1% lecithin	106	26	5.1	10.0	308	62	93
	50 mg. dianisylhexene/lb. ration	142	51	7.8	28.0	765	83	148
	50 mg. dianisylhexene +1% lecithin/lb. ration	204	54	7.6	29.0	570	73	159
5-14	Control+1% lecithin	134	35	6.1	13.0	304	70	96
	50 mg. dianisylhexene/lb. ration	238	166	15.2	72.0	1208	305	410
	50 mg. dianisylhexene +1% lecithin/lb. ration	189	141	13.2	72.0	2321	268	386
5-24	Control+1% lecithin		35		17.0	402	65	127
	50 mg. dianisylhexene/lb. ration		250		62.0	3104	139	439
	50 mg. dianisylhexene +1% lecithin/lb. ration		189		62.0	4685	225	515
5-25[1]	Control+1% lecithin					635	47	
	50 mg. dianisylhexene/lb. ration					4952	227	
	50 mg. dianisylhexene +1% lecithin/ration					9757	145	
5-27	Control+1% lecithin					340	57	
	50 mg. dianisylhexene/lb. ration					3548	165	
	50 mg. dianisylhexene +1% lecithin/ration					3681	174	
5-28	Control+1% lecithin					281	52	
	50 mg. dianisylhexene/lb. ration					532	47	
	50 mg. dianisylhexene +1% lecithin/lb. ration					295	151	
6-1	Control+1% lecithin					369	57	
	50 mg. dianisylhexene/lb. ration					369	59	
						369	64	

[1] Estrogens discontinued after this date.

these conditions. Therefore only the free cholesterol and total fatty acids resulting from the use of the estrogens are reported after this date. The data in the second section of Table 2 were obtained to determine the use of lecithin as a carrier for the estrogens as well as to determine the duration of these chemical changes after discontinuing the estrogen feeding.

Table 3-A records the average free cholesterol and total fatty acid content of the blood of twelve-week-old fryers of mixed sex which had

been fed the control ration supplemented with various estrogens. Lecithin was added to certain of these rations to determine its effect on the utilization of the estrogens. The total fatty acid values in Table

TABLE 3. FREE CHOLESTEROL AND TOTAL FATTY ACID CONTENT OF BLOOD

A. 12-week-old fryers of both sexes

Free cholesterol							Total fatty acids						
3-18	3-20	3-22	3-24	3-26	3-28	3-30	3-18	3-20	3-22	3-24	3-26	3-28	3-30
Control	mg./100 ml. blood)								(mg./100 ml. blood)				
64.1	64.0	65.6	66.8	62.9	70.0	76.8	299	322	331	364	356	295	304
100 mg. triphenylchloroethylene/lb. ration													
57.0	62.9	81.7	86.7	107.0	222.0	263.0	251	710	1654	1357	3420	3431	3251
200 mg. triphenylchloroethylene/lb. ration													
59.8	93.5	110.2	120.6	138.5	213.0	278.0	269	680	1774	1895	3731	3226	4626
300 mg. triphenylchloroethylene/lb. ration													
62.9	102.1	119.0	173.0	183.3	256.0	263.0	312	562	1237	3352	3278	4332	5103
40 mg. dienestrol diacetate/lb. ration													
62.2	69.1	63.8	63.6	69.7	62.2	67.0	303	281	310	300	404	352	266
60 mg. dienestrol diacetate/lb. ration													
63.9	62.2	62.7	66.4	68.0	71.3	71.4	325	—	285	308	359	305	229
10 mg. dianisylhexene/lb. ration													
70.9	70.4	67.5	79.4	68.8	73.5	83.3	387	319	373	370	334	234	275
30 mg. dianisylhexene/lb. ration													
65.8	64.1	110.0	63.3	—	178.0	111.0	328	417	1508	482	450	1669	923
40 mg. dianisylhexene/lb. ration													
71.4	71.9	88.5	110.3	120.0	164.0	180.0	340	377	679	1118	1443	2323	2874
50 mg. dianisylhexene/lb. ration													
66.0	78.3	89.0	122.1	192.0	310.0	350.0	236	768	875	1361	2088	2486	3957
50 mg. dianisylhexene/lb. ration													
61.3	88.5	128.0	148.0	173.0	278.0	345.0	219	1319	2819	6016	8350	9530	9468
Ration plus 1% lecithin													
69.2	65.0	73.4	78.3	50.1	98.1	92.6	243	231	279	325	251	241	294

B. 12-week-old chicken fryers

	Total fatty acids				
	10-10	10-12	10-14	10-17	10-21
		(mg./100 ml. blood)			
Control	236	244	274	353	370
50 mg. dianisylhexene/lb. ration	222	816	1135	4043	7316
50 mg. dianisylhexene/lb. ration plus 5 grams soybean lecithin	275	512	1637	3521	5376
100 mg. triphenylchloroethylene/lb. ration	265	338	836	1994	3074
100 mg. triphenylchloroethylene/lb. ration plus 5 grams soybean lecithin	272	763	983	3077	3062

C. mature market hens

Free cholesterol			Total fatty acids		
12-10	12-16	1-4	12-10	12-16	1-4
(mg./100 ml. blood)			(mg./100 ml. blood)		
Control					
60.1	60.3	59.1	810	702	704
50 mg. dianisylhexene/lb. ration					
62.7	131.8	130.5	718	1820	4974

D. old cocks

Free cholesterol					Total fatty acids				
5-4	5-5	5-6	5-8	5-14	5-4	5-5	5-6	5-8	5-14
(mg./100 ml. blood)					(mg./100 ml. blood)				
Basal plus 1% lecithin									
93.5	120.0	110.0	87.7	96.2	217	226	226	353	522
15 mg. diethylstilbestrol injected in neck in oil solution May 4 plus 50 mg. dianisylhexene/lb. ration									
86.2	95.2	175.0	200.0	286.0	328	216	1229	2405	7392
50 mg. dianisylhexene/lb. ration									
96.2	90.1	106.0	100.0	217.0	208	276	393	384	2376

3-B measure the relative effect of soybean oil and a combination of soybean oil and soybean lecithin on the utilization of triphenylchloroethylene and dianisylhexene. Table 3-C gives blood data for mature market hens and Table 3-D for old cocks.

To compare the relative efficiency of implanted pellets and orally administered estrogen, young fryers were divided into three lots and fed as follows: One lot received the unsupplenented basal ration, the second lot received implanted pellets containing 15 mg. diethylstilbesterol each, and the third lot received dianisylhexene mixed with the feed at a dosage level of 50 mg. per pound of feed. These rations were fed for a period of 34 days after which the estrogen supplements

TABLE 4. ANALYSES OF BLOOD OF TWELVE-WEEK-OLD FRYERS RECEIVING ESTROGENS ADMINISTERED AS PELLET IMPLANTS AND AS A COMPONENT OF THE FEED

Feed	5-30	6-1	6-3	6-6	6-13	6-20	6-27*	7-2	7-5	7-7	7-9
					(mg./100 ml. whole blood)						
					Free Cholesterol						
Basal Ration (Control)	79	75	71	80	105	106	85	121	74	93	82
Diethylstilbestrol pellet implants in neck	77	78	72	87	109	128	76	86	61	68	82
50 mg. dianisylhexene/lb. ration	71	79	93	294	444	506	407	328	281	123	93
					Total Fatty Acids						
Basal Ration (Control)	388	355	295	369	355	340	455	366	322	195	279
Stilbestrol pellet implants in neck	369	413	369	413	399	370	277	674	277	274	224
50 mg. dianisylhexene/lb. ration	325	606	1493	4376	2833	5544	2212	2212	2475	374	250

* Estrogens discontinued after this date.

TABLE 5. ANALYSES OF DEHYDRATED FAT-FREE CHICKEN BONES AT END OF 17-DAY FEEDING PERIOD

Feed	Total Ash	Ca	P	Magnesium
	%	%	%	%
Basal	55.0	38.4	17.4	.483
Basal +1 % lecithin	50.8	31.0	16.7	.447
40 mg. dianisylhexene/lb. ration	55.0	38.3	17.6	.605
60 mg. dianisylhexene/lb. ration	53.0	36.2	17.1	.487
50 mg. dianisylhexene/lb. ration plus 1 % lecithin	48.6	36.8	17.0	.472

were discontinued and all lots were fed the unsupplemented basal ration for an additional 7 days. The resulting analyses recorded in Table 4 indicate that in this determination the implanted pellet was utilized very slowly and much less effectively than a subcutaneous injection of an oil solution of the estrogen or the use of a mixed feed containing an oil solution of the estrogen. These results also indicate that the estrogen apparently is not stored in the body for any considerable period of time after estrogen feeding is discontinued.

During the progress of this investigation it was observed that the incidence of broken bones was greater in the estrogen-fed lots. Therefore the bone ash of the fat-free dehydrated bones of these chickens was determined on several occasions. The results on such series are recorded in Table 5 together with the calcium, phosphorus, and magnesium percentages of the ash.

To further test the possible changes in the ash content over a longer period of time the ash of bones from chickens receiving 50 mg. of dianisylhexene was determined each week for a five-week period, with the following percentages: 49.88, 49.44, 47.71, 47.90, and 48.70, respectively.

Turkeys

The effect of orally administered estrogens upon the total fatty acid and free cholesterol levels of turkey blood was studied in a similar

TABLE 6. CHOLESTEROL AND FATTY ACID CONTENT OF TURKEY BLOOD

Feed	Free cholesterol					Total fatty acids				
	1st	8th	15th	22nd	29th	1st	8th	15th	22nd	29th
	(mg./100 ml. blood)									
	A. Twelve-week-old									
Basal Ration	65	75	103			276	306	309		
50 mg. dianisylhexene/lb. ration	71	226	206			275	1206	5603		
	B. Twenty-week-old									
Basal Ration	81	99	95	102	81	365	450	517	383	440
40 mg. dianisylhexene/lb. ration	71	260	281	342	415	308	1987	4801	6380	6836
60 mg. dianisylhexene/lb. ration	73	253	309	309	501	361	3156	5271	7178	9135
80 mg. dianisylhexene/lb. ration	78	253	303	308	503	346	4261	6325	8348	9150

TABLE 7. TOTAL FATTY ACID IN BLOOD OF 12-WEEK-OLD
TURKEYS AFTER 3 WEEKS ON FEED

	Basal Ration	50 mg. dianisyl- hexene per lb. feed	100 mg. triphenyl- chloroethylene per lb. feed	200 mg. triphenyl- chloroethylene per lb. feed
mg./100 ml. blood	406	5703	1409	3702

manner. The results recorded here are the average analyses of the blood of young turkeys ranging in age from 9 to 24 weeks. Table 6-A records the free cholesterol and total fatty acid values of the blood of two lots of turkey broilers which were 10 and 11 weeks of age at the beginning of the three-week premarket fattening period. One lot was fed the unsupplemented basal ration and the other the basal ration supplemented with estrogen as indicated. Table 6-B records both the free cholesterol and total fatty acids levels of the blood of 20-week-old turkeys fed different levels of dianisylhexene during a four-week feeding period. Table 7 records the average total fatty acid content of the blood of 12-week-old turkey broilers which had been fed one level of dianisylhexene and two levels of triphenylchloroethylene during a three-week fattening period.

SUMMARY

Oral administration of estrogens was more effective with poultry than pellet implants. Of the blood components the fatty acid, choles-

terol and lipoid phosphorus showed phenomenal changes following the administration of estrogens. Injections of oil solutions of estrogens produced chemical changes within a few hours. The maximum change with either pellet implant or oral administration was indicated within a week. Blood constituents returned to near normal within 3 to 4 days after estrogen administration was discontinued. Although the bones of these fowl seemed to be more fragile, there was no significant change in tibia ash analyses.

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AN ATTEMPT TO EXPLAIN THE ANOMALOUS ACTION OF LUGOL'S SOLUTION IN EXOPHTHALMIC GOITER[1]

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IN A SERIES of investigations extending over a number of years we have followed a certain working hypothesis, although we realize that other hypotheses have been followed by other investigators. We consider a goiter a thyroid gland enlarged by compensatory hypertrophy and therefore capable of producing more thyroglobulin than the normal thyroid. The distinction of diffuse and adenomatous goiter often fails due to the finding of minute or hidden adenomas in goiters previously classified as diffuse. Kocher, of Berne, Switzerland, would not allow iodine used on his goiter patients for fear of making them all hyperthyroid but Plummer, of the Mayo Clinic, dosed his patients with Lugol's solution and demonstrated its anomalous action in lowering the basal metabolic rate in exophthalmic goiter.

We have shown that thyroglobulin from untreated goiters has a low thyroxine content (Cavett, Rice and McClendon, '35). One of these thyroglobulins from a goiter contained only 0.0046% thyroxine whereas thyroglobulin from as nearly normal thyroids as we could obtain at autopsy contained about 0.3% thyroxine. When these thyroglobulins were fed to rats, at the dose of 280 micrograms of thyroxine iodine per 100 sq. cm. of body surface, they increased the metabolic rate in proportion to their thyroxine content (McClendon, Foster and Cavett, '41). The presence of live thyroid tissue is not necessary in iodinating thyroglobulin. When one of these goitrous thyroglobulins was dissolved in physiological salt solution and stirred with iodine at body temperature and pH for 20 hours its thyroxine iodine content was increased from 0.06% to 0.342% (McClendon and Foster, '44, a). Its effect on the metabolic rate of rats was also increased (McClendon and Foster, '44, b). We must conclude, therefore, that there is not enough elemental iodine in a goiter to fully iodinate the thyroglobulin, and that the increased metabolic rate in exophthalmic goiter is due to release of *large amounts* of thyroglobulin whose *total* thyroxine content is greater than normal.

Since the action of Lugol's solution is to raise the thyroxine content of goitrous thyroglobulin (Cavett, Rice and McClendon, '35) its action in lowering the basal metabolic rate must be sought outside the thyroid, perhaps in the anterior pituitary. We have not attempted to determine the thyroxine content of the anterior pituitary, but since thyroxine seems to be present chiefly in protein combination in the body we determined the protein bound iodine in the pituitary.

It is well known that an increase in protein bound iodine *arising from the thyroid* will inhibit the production of thyrotropin by the pituitary. It seems reasonable that this protein bound iodine must reach the pituitary in order to have its effect. If protein bound iodine were *synthesized within the pituitary itself*, how could the pituitary distinguish between that and the supply from the thyroid gland? Perhaps the protein bound iodine synthesized within the pituitary might inhibit the pituitary's secretion of thyrotropin. Perhaps the protein bound iodine synthesized within the pituitary would not diffuse out and certainly the determinations on sheep given below suggests that it accumulates there. If this were true it would explain the effect of Lugol's solution in inhibiting the thyroid. The Lugol's solution would first iodinate protein in the pituitary, this protein would inhibit the thyrotropin production. The lessened amount of thyrotropin would abolish the stimulating effect on the thyroid, and the resting thyroid would not release thyroglobulin into the circulation, but we wished to put this hypothesis to the test. Due to the great variability in the diet of dogs we decided to use a couple of sheep (ewes) which a farmer assured us were twins and had always grazed in the same pasture. Although these sheep were 7 years old one could not be out of sight of the other without baaing continuously. One of these sheep was fed 100 milligrams potassium iodide a day in the form of a sandwhich. Some of the iodide changed to iodine and colored the starch of the bread blue. The other was given 3 grams of thiouracil a day in the form of a sandwich, but refused it from the third day onward so it was given by means of a tube and plunger. The pituitary of the sheep fed 100 mg. potassium iodide a day for 66 days contained 263 per cent as much protein bound iodine as the pituitary of the sheep receiving 3 grams a day of thiouracil (deracil) for 41 days (McClendon and Foster, '47). The weight of the potassium iodide fed pituitary was 1.625 grams and that of the thiouracil fed 1.18 grams. Due to the expense of sheep we decided to use chickens for continuing these experiments.

MATERIAL AND METHODS

More than 200 New Hampshire Red chickens, 12 weeks old and weighing about 4 pounds each were used. Seventy-one were used for controls, 74 were given 4 cc. of Lugol's solution per gallon of drinking water and 71 were fed 0.2% thiouracil in their feed to deplete their bodies of elemental iodine. Two experiments were run, one of 2 weeks and one of 3 weeks. At the end of these

experiments the chickens were killed, weighed, and pituitary and thyroid glands removed, weighed, and after the inorganic iodine was extracted, preserved in vials for protein bound iodine analysis.

The thyroid glands are paired and lie in the thorax, mesially to the jugular vein, in the angle formed by the subclavian and common carotid arteries. They were removed after first removing the crop. The pituitaries were removed by operating from the hard palate after removal of the lower jaw.

The inorganic iodine was extracted 5 minutes with cold methanol and 5 minutes with cold acetone in the 3 week experiments, but for fear that this was not sufficient it was extracted an additional 2 months with cold acetone

TABLE 1. MEAN VALUES OF PROTEIN BOUND IODINE IN PITUITARY AND THYROID OF CHICKS FED 0.2% THIOURACIL

Weeks of feeding	Number of chicks	Body wt. lbs.	Pituitary						Thyroid		
			Wt. mg.	S.D.	St. error of mean	Prob. error of mean	Iodine		Milligrams		% Iodine dry
							γ	γ per gm.	wet	dry	
3	12	3.38	15.5				0.041	2.63	518	87	0.025
3	12	3.47	13.5				0.038	2.78	530	89	0.024
3	24	3.42	14.5	3.39	0.69	0.47	0.039	2.70	524	88	0.025
2	10	4.09	19.5				0.032	1.64	437	80	0.018
2	10	4.24	21.0				0.062	2.96	452	98	0.021
2	10	3.49	17.1				0.048	2.81	360	75	0.019
2	10	4.04	14.6				0.037	2.54	393	66	0.020
2	10	3.95	20.9				0.051	2.44	437	99	0.017
2	50	3.96	18.6	5.58	0.79	0.53	0.046	2.47	416	83	0.019
2 and 3	74	3.79	17.3				0.044	2.53	451	85	0.021

TABLE 2. MEAN VALUES OF PROTEIN BOUND IODINE IN PITUITARY AND THYROID OF CONTROL CHICKS

Weeks of feeding	Number of chicks	Body wt. lbs.	Pituitary						Thyroid		
			Wt. mg.	S.D.	St. error of mean	Prob. error of mean	Iodine		Milligrams		% Iodine dry
							γ	γ per gm.	wet	dry	
3	12	3.70	18.4				0.048	2.63	154	37	0.175
3	10	3.89	17.7				0.048	2.71	144	32	0.257
3	22	3.79	18.1	6.33	1.35	0.91	0.048	2.67	149	35	0.216
2	10	4?	15.3				0.054	3.52	171	55	0.179
2	10	4?	14.6				0.042	2.87	181	51	0.199
2	10	4?	15.6				0.066	4.24	188	51	0.215
2	10	4?	19.9				0.056	2.81	172	48	0.258
2	9	4?	19.7				0.051	2.60	199	54	0.219
2	49	4?	17.0	5.00	0.71	0.48	0.054	3.17	182	52	0.214
2 and 3	71	4?	17.3				0.052	3.01	172	47	0.215

in the 2 week experiments. The remainder was considered protein bound iodine (McClendon and Foster, '44, c). The 2 month extraction showed no lower values of protein bound iodine except in those chickens fed Lugol's solution for only 2 weeks. But one would expect a less effect of Lugol's solution in 2 weeks than in 3 weeks. Therefore we believe that the extraction of inorganic iodine was complete within the limit of error of the methods. The iodine determinations were made by the method of McClendon and Bratton ('38).

RESULTS

From Tables 1, 2 and 3 it may be seen that thiouracil markedly increased both wet and dry weight and lowered the protein bound iodine in the thyroid to 10 per cent of its normal value, whereas Lugol's solution did little to the wet and less to the dry weight but more than doubled the protein bound iodine in the thyroid. The effect on the pituitary is much less and therefore statistical methods were applied to it. It was thought that for strict application of statistics the 3 week and 2 week experiments should be treated separately.

In the 3 week experiments the mean pituitary weight in the thio-

TABLE 3. MEAN VALUES OF PROTEIN BOUND IODINE IN PITUITARY AND THYROID OF CHICKS DRINKING 4 .CC. LUGOL'S PER GAL.

Weeks of feeding	Number of chicks	Body wt. lbs.	Pituitary							Thyroid		
			mg. Wt.	S.D.	St. error of mean	Prob. error of mean	Iodine		Milligrams		% Iodine dry
							γ	γ per gm.	wet	dry	
3	10	3.79	16.8				0.075	4.45	220	60	0.682
3	11	3.53	17.3				0.063	3.63	177	49	0.660
3	21	3.65	17.1	5.77	1.26	0.85	0.069	4.01	198	54	0.671
2	10	4.04	22.7				0.075	3.31	220	62	0.724
2	10	3.94	26.4				0.104	3.94	196	48	0.325
2	9	3.53	18.3				0.120	6.56	186	47	0.317
2	10	3.79	20.2				0.062	3.07	234	62	0.306
2	11	3.84	18.9				0.045	2.40	217	55	
2	50	3.83	21.3	7.49	1.06	0.71	0.080	3.75	209	55	0.418
2 and 3	71	3.78	20.1				0.077	3.81	204	55	0.502

uracil fed chickens is 14.49 ± 0.47 mg. whereas that of the controls is 18.05 ± 0.91 mg. The standard error of the difference of these two means is 2.347, and the probability of this not being due to chance is 50 to 1. The mean weight of the pituitary in the Lugol's fed chickens is 17.08 ± 0.85 and the standard error of the difference from the control is 0.45. The probability of this difference being due to chance is about 1 to 1 and therefore it is doubtful that Lugol's solution affected the weight of the pituitary.

In the 2 week experiments the mean weight of pituitary of the thiouracil fed chickens is 18.61 ± 0.53 mg. and that of the controls 16.96 ± 0.48 mg. The standard error of the difference of these means is 1.55. The probability of this not being due to chance is about 7 to 1 but the difference is in opposite direction to the 3 week experiment. It therefore seems doubtful that thiouracil had any effect on the weight of the pituitary. The mean pituitary weight of the Lugol's fed chickens is 21.31 ± 0.71 and the standard error of the difference between this and the control is 1.7 and the probability of this difference not being due to chance is about 1483 to 1. But since there was no difference in the feeding of the control group in the 2 and 3 week experiments it is per-

missible to compare the 2 week Lugol's fed chickens with the 3 week controls in which case the standard error of the difference of means is 1.71 and the probability of the difference not being due to chance is 16 to 1. Whereas the data suggest that Lugol's solution increases the weight of the pituitary gland they do not prove the point.

Although the glands of the 216 chickens were weighed individually the protein bound iodine in a single pituitary was about 0.05 microgram and too small for accurate determination. Therefore the mean value of about 10 glands was determined by analyzing the group and dividing by the number of glands. The number of groups is not sufficient for statistical analysis but the large differences as shown in

TABLE 4. MEAN VALUES OF PROTEIN BOUND IODINE IN THE PITUITARY GLANDS OF NORMAL CHICKENS COMPARED WITH THOSE GIVEN THIOURACIL OR LUGOL'S SOLUTION

Treatment	Weeks of exp.	No. of chicks	γ	γ per gm.	Per cent of control	
					γ	γ per gm.
Thiouracil	3	24	0.039	2.70	75	90
	2	50	0.046	2.47	87	82
	2 and 3	74	0.044	2.53	85	84
Control		71	0.052	3.01		
Lugol's	3	21	0.069	4.01	133	133
	2	50	0.080	3.75	154	125
	2 and 3	71	0.077	3.81	148	127

Table 4 are impressive. Since there was no difference in the feeding of the controls in 2 as compared with the 3 week experiments they have been combined.

Thiouracil reduced the protein bound iodine in the pituitary gland to 85 per cent of its normal value and Lugol's solution increased it to 148 per cent of normal. If we take into consideration the weight of the pituitary gland and calculate the micrograms of protein bound iodine per gram of gland, thiouracil reduced it to 84 per cent of normal and Lugol's solution increased it to 127 per cent of normal.

We may conclude that Lugol's solution increases the protein bound iodine of the pituitary gland and that is a possible explanation of its action in exophthalmic goiter. For this to be true it is not necessary that Lugol's solution should always lower the basal metabolic rate, but only in those cases where a high rate is due to a high liberation of thyrotropin by the pituitary gland.

That there may be other mechanisms of the action of Lugol's solution is freely admitted, but they have been reviewed by Rawson ('47) and others and space does not admit of their adequate evaluation.

SUMMARY

The thyroid and pituitary glands of 216 twelve week old chickens were weighed and analyzed for protein bound iodine. One third had been given 0.2 per cent thiouracil in the feed, one third had 4 cc. of Lugol's solution added per gallon of drinking water and one third were used as controls.

Thiouracil markedly increased both wet and dry weight of the thyroid glands and reduced the protein bound iodine to 10 per cent of its normal value. The protein bound iodine of the pituitary glands was reduced to 85 per cent of its normal value.

Lugol's solution increased the protein bound iodine of the thyroid 133 per cent and of the pituitary 48 per cent. It seems probable that local action of this protein bound iodine in the pituitary might inhibit the release of thyrotropin and explain the anomalous action of Lugol's solution in exophthalmic goiter.

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METABOLISM OF THE STEROID HORMONES:
THE ISOLATION OF ETIOCHOLANEDIOL-3(α), 17(α) FROM HUMAN URINE[1]

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ETIOCHOLANEDIOL-3(α), 17(α) has been isolated from the urine of two patients. One patient was a male with Addison's disease (Patient A) who received 1350 mg. of dehydroisoandrosterone. The other patient was a young woman (Patient B) who had symptoms of virilism and a high excretion of androgens and 17-ketosteroids (Schiller *et al.*, 1945a).

No isolation of etiocholanediol-3(α), 17(α) has been reported at the present time. A small amount of a non-ketonic compound which appeared to be an impure sample of etiocholanediol-3(α), 17(α) was reported by Dorfman *et al.* (1939). Butenandt (1937) described the isolation of this diol from a urinary extract which had been reduced with aluminum amalgam. It was not found in urinary extracts not treated with aluminum amalgam.

<div align="center">EXPERIMENTAL</div>

Patient A

The urine was collected for 3 days during oral administration of 450 mg. per day of dehydroisoandrosterone.[2] The non-ketonic digitonic soluble fraction was prepared from acid hydrolyzed urine by a method previously described (Schiller, Dorfman, and Miller, 1945). This fraction was acetylated and chromatographed on aluminum oxide (Merck-Brockmann). Five mg. of a compound (M.P. 124–127°C.) was obtained by elution with 50% benzene-petroleum ether. The melting point of this compound was not depressed when mixed with a sample of etiocholanediol-3(α), 17(α) diacetate, melting point 126–127°C. prepared by reduction of etiocholanol-3(α)-one-17 with sodium and isopropyl alcohol and subsequent acetylation.

Patient B

The neutral non-ketonic digitonin non-precipitable fraction was prepared from a benzene extract of 74 liters of unhydrolyzed urine. The material was chromatographed 3 times on aluminium oxide. 3 mg. crystals m.p. 232–233°C. were obtained by elution with 0.1% absolute ethanol in benzene. The melting point was not depressed by mixture with etiocholanediol-3(α), 17(α) m.p. 232°–234° prepared from etiocholanol-3(α)-one-17. The material was acetylated yielding a product m.p. 125°–126°C. When mixed with etiocholanediol-3(α), 17(α), diacetate m.p. 126°–127° prepared from etiocholanol-3(α)-one-17, the mixture melted at 125°–127°.

The melting points[3] reported for etiocholanediol-3(α), 17(α) are 232°C. (Butenandt, Tscherning, and Dannenberg, 1937) and 236–236.5°C. (corr.) (Ruzicka, Goldberg, and Bosshard, 1937). Ercoli (1938) reported a melting point of 124.5–125.5°C. (corr.) for the diacetate.

<div align="center">DISCUSSION</div>

Whether etiocholanediol-3(α), 17(α) arose as a metabolite of dehydroisoandrosterone cannot be decided at this time. The yield of the diol was exceedingly small. It is known, however, that dehydroisoandrosterone is metabolized to etiocholanol-3(α)-one-17 (Mason and Kepler, 1945; Miller, Dorfman, Miller). It is possible that the diol represents a further reduction of etiocholanol-3(α)-one-17.

The etiocholanediol-3(α), 17(α) was isolated from the extract prepared from the unhydrolyzed urine of the woman with symptoms of virilism. The unhydrolyzed urine extracts contained half of the 17-ketosteroids. It would be expected that the extract prepared from the residual urine after acid hydrolysis will contain additional quantities of the diol. This fraction has not been studied at the present time.

<div align="center">SUMMARY</div>

Etiocholanediol-3(α), 17(α) has been isolated from the urine of a male with Addison's disease who received dehydroisoandrosterone and from the urine of a young woman with symptoms of virilism.

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[3] All melting points were taken with the Fischer-Johns apparatus and are uncorrected.

HYALURONIDASE LEVELS OF RAT TESTES AS RELATED TO AGE, HYPOPHYSECTOMY AND CRYPTORCHIDISM[1]

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SINCE the discovery of the enzyme hyaluronidase in mammalian testes, one of the problems in which we have been interested has been to determine by what means the enzyme level in the testes might be altered. Previous to identification of hyaluronidase with the "spreading factor," Sprunt, *et al.* (1939) showed that the latter was present in the testes of immature rabbits and cryptorchid rats in amounts less than in normal adults. In a preliminary note (Leonard and Perlman, 1946) it was reported that hyaluronidase, determined by the turbidimetric method, was lower in immature, cryptorchid, and hypophysectomized rats. The details of these experiments are reported here.

METHODS

Determinations of hyaluronidase were made according to the previously described turbidimetric method (Leonard, Perlman and Kurzrok, 1946). The units of enzyme are expressed as turbidity reducing units (TRU). The same batch of hyaluronic acid was used throughout these experiments. Assays also were made using a modification of the rat-ova test (Leonard and Kurzrok, 1946) in which a comparison of the time required to disperse the follicle cells as related to dosage was used as a measure of relative activity. Testes homogenates were prepared in acetate buffer (pH 6) for the turbidimetric tests and in Ringer's solution when the rat-ova test was used. Testes of several rats were pooled in making certain of the extracts in order to obtain sufficient material for assay. Histological examination of the testes from rats were made on samples representative of the several experiments.

RESULTS

Experiments were performed to determine the relationship of age and testes weight to the enzyme level. By the turbidimetric method it was found that traces of enzyme up to 2 TRU were present in testes of rats 21–30 days old and with increasing age there was an increase up to 10 TRU per gram of tissue (Table 1). Because the variation in size of the rats from different litters at any given age would be reflected in

the size of the testes, it seemed best to correlate testis weight with enzyme level. Since all testes extracts were prepared so that 1 gram of tissue was equivalent to 1 cc. of extract, values of less than 2 units could not be obtained, although in these instances traces of activity were observed. No attempt was made to concentrate the enzyme as it seemed sufficient to show that a decreased amount of hyaluronidase was present in the small testes of the youngest rats. In order to prove that the traces of activity were real and not an artifact, aliquot samples were heated to destroy the enzyme and in every case assay of the heated material indicated no activity.

The results of similar assays but using the dispersal time of the

TABLE 1. HYALURONIDASE ACTIVITY OF TESTES AS A FUNCTION OF AGE AND TESTIS WEIGHT IN THE RAT

Age group (days)	No. of assays	No. of rats	Testes weight per rat (gm.)	TRU per gram of testis
90 and over	7	15	1.80–3.35	10–11
50	4	6	1.40–1.56	10
32–44	3	13	0.67–1.12	5–7
21–30	3	14	0.42–0.45	<2–2

follicle cells indicated the same trend in the appearance of the enzyme with increasing development of the testes (Table 3). The concentration of enzyme was too low to be detected in the 26-day old rats and had about reached its adult level in 50-day old rats.

Histological examination of the testes revealed only the early stages of spermatogenesis in the 26-day old rats. Those at 34 days showed an increase in tubule size but no marked difference in the stages of spermatogenesis. At 50 days the tubules were still larger, and possessed spermatozoa as well as spermatids in great numbers.

In another experiment adult rats, weighing not less than 250 grams, were made cryptorchid by severing the gubernaculum and anchoring the testes to the abdominal wall. Assay of the enzyme concentration five days later showed no decrease in the hyaluronidase level but by the 12th day a low concentration was found beyond which no further drop occurred on either 21 or 30 days (Table 2). The rat-ova test indicated a slight drop in enzyme by 10 days and no detectable enzyme on the 21st day (Table 3).

Histologically, the testes of 5-day cryptorchid rats revealed slightly smaller tubules, loss of many sperm, and some degenerating epithelium. By the 12th day the tubules showed a characteristic marked degeneration, the germinal epithelium in some tubules, however, was intact although no fully formed sperm were present. No further degenerative changes were observed in the testes of rats on the 21st day after operation.

TABLE 2. HYALURONIDASE ACTIVITY IN RAT TESTES FOLLOWING CRYPTORCHIDISM AND HYPOPHYSECTOMY

Exper.	No. of assays	No. of rats	Testes weight per rat (gm.)	TRU per gram of testes
Normal adults	7	15	1.80–3.35	10–11
Cryptorchid (days)				
5	1	4	2.20	11
12	1	3	1.50	3
21	1	2	1.40	4
30	2	4	1.2–1.6	3
Hypophysectomy (days)				
10	9	15	1.6–2.5	11.7 (10–15)
20	3	6	1.0–1.2	8.3 (7–10)
24	5	9	0.7–0.8	3.8 (1–6)
29	1	1	0.40	4
30	1	1	0.40	4
62	1	7	0.34	3

Adult rats over 250 grams body weight were hypophysectomized and the testes assayed at intervals from 10 to 62 days later. It was noted that the fall in enzyme level was slight up to 20 days post-operatively but dropped rapidly by 24 days (Table 2). Beyond this the enzyme level constantly remained low. The results from the rat-ova test were similar in that only a very small drop in enzyme level

TABLE 3. HYALURONIDASE FROM RAT TESTES UNDER DIFFERENT EXPERIMENTAL CONDITIONS ASSAYED BY THE RAT-OVA METHOD

Exper.	Testes weight per rat (gm.)	Testes extract (gm. equiv.)	Time of follicle cell dispersal (hours)
Normal Adults	2–3.2	.05–.10	$\frac{3}{4}$–1
26 days	0.4	0.20	0 dispersal
26 days	0.4	0.20	0 dispersal
34 days	0.7	0.20	2; $2\frac{1}{4}$; $2\frac{1}{4}$
50 days	1.6	0.20	$1\frac{1}{2}$; $1\frac{1}{2}$
50 days	1.4	0.20	$\frac{1}{2}$, $\frac{1}{2}$
		0.10	$\frac{3}{4}$, $\frac{3}{4}$
Cryptorchid			
10 days		0.20	$\frac{1}{2}$; $\frac{3}{4}$
		0.10	1; $1\frac{1}{2}$
21 days		0.20	0 dispersal
Hypophysectomy			
10 days		0.20	$\frac{1}{2}$; $\frac{3}{4}$
		0.10	1; $1\frac{1}{2}$
20 days		0.20	$\frac{1}{2}$; $\frac{1}{2}$
		0.10	$\frac{3}{4}$; $\frac{3}{4}$
24 days		0.20	$4\frac{1}{2}$; $3\frac{1}{2}$
24 days		0.20	0 dispersal

was obtained up to the 20th day but a marked drop was observed by the 24th day.

Histologically, the testis tubules of 10-day hypophysectomized rats showed relatively little change. Many sperm and spermatids were present, although the tubules appeared smaller. By the 20th day the tubules contained many secondary spermatocytes, only a few spermatids, and no sperm. Testes examined on the 24th day showed no spermatids, and marked reduction in the depth of the germinal epithelium.

<div align="center">DISCUSSION</div>

These results indicate that some enzyme appears in the germinal cells of the testes in the absence of fully developed sperm. This was seen in the testis of immature, cryptorchid, and hypophysectomized rats. In the last two conditions some germinal elements were seen in a number of the tubules although widespread injury was observed in most of them. These remaining cells might account for the trace of activity observed.

Where normal and presumably maximum numbers of spermatozoa were present in the testes, the maximum amount of hyaluronidase was obtained. This might be attributed to a higher content of enzyme in the cells or to the greater proportion of enzyme-producing cells to the non-germinal elements in the gonads. It seems unlikely that other cells would produce the enzyme. For example, the interstitial cells of cryptorchid testes were normal and those of the hypophysectomized rat were atrophied yet the enzyme concentration was approximately the same. It is conceivable, however, that the Sertoli cells might also contribute some enzyme.

Sprunt, et al. (1939) showed that the spreading factor was decreased in the testes of immature rabbits and cryptorchid rats. They stated that the amount of spreading factor is related to the amount of spermatogenesis. Since it has been shown that testes spreading factor and hyaluronidase are the same or closely related substances our results confirm those of the above investigators. Swyer (1947) however was unable to demonstrate any hyaluronidase in the testes of cryptorchid or prepubertal rabbits.

Our original problem was to determine what factors might affect the level of hyaluronidase in the cells producing the enzyme and it seemed that the usual metabolic derangements accompanying hypophysectomy would affect the metabolism of these cells and thereby the production of the enzyme. However, the failure to obtain any decrease in enzyme 10 days after hypophysectomy and only slightly at 20 days seems to indicate that alteration of the enzyme level in the testes without severe injury to the germinal epithelium will be very difficult if not impossible.

SUMMARY

Hyaluronidase determinations were made on rat testes by the turbidimetric method and the rat-ova test in animals of varying ages, and following cryptorchidism and hypophysectomy. A gradual increase in the amount of enzyme per gram of tissue was noted between 21 days of age and sexual maturity. After experimental cryptorchidism, the hyaluronidase level was low by the 10th day following the operation and after hypophysectomy it was low by the 24th day. Enzyme levels were correlated directly with the degree of development of the germinal epithelium. All of the enzyme of the testes was not accounted for by mature sperm since it was detected in testes devoid of sperm.

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THE PATHOLOGY OF THE ADRENAL GLAND IN ADDISON'S DISEASE WITH SPECIAL REFERENCE TO ADRENOCORTICAL CONTRACTION

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"CYTOTOXIC" necrosis, atrophy or contraction of the adrenal cortex associated with functional insufficiency has been recognized as a clinicopathologic entity for many years (Barker, 1929; Guttman, 1930; Duffin, 1943). It is also well known that the incidence of adrenocortical contraction as a cause of Addison's disease now equals or exceeds that of tuberculosis of the adrenal. Nevertheless, the continuing uncertainty among endocrinologists about the essential nature of the process is evidenced by a recent report (Rogoff, 1944) in which the lesions of contraction were confused with ischemic lesions of the adrenal.

This study was stimulated by the receipt at the Army Institute of Pathology, from the beginning of World War II through 1946, of pathologic material from 15 patients who exhibited adrenocortical contraction at autopsy; during the same period only 10 cases of Addison's disease resulting from tuberculosis or fibrocaseous destruction were accessioned. The time at which the first outbreak of epidemic hepatitis reached its height during the war coincided with the period during which many of the examples of adrenocortical contraction appeared. During a discussion, Dr. Balduin Lucké, who did extensive investigation on epidemic hepatitis, pointed out that the patterns of postnecrotic parenchymal collapse and secondary superimposed nodular "regeneration" and proliferation, which characterized the hepatic lesions, were duplicated in the adrenal. The similarity between adrenal contraction and certain hepatic processes had been noted before (Wells, 1930; Kiefer, 1927; Duffin, 1943).

Prior to World War II only 6 cases of adrenocortical contraction had been accessioned at the Institute, in contrast to 21 cases of Addison's disease caused by tuberculosis. The relative incidence of tuberculosis and contraction in the cases of Addisons' disease in the files of the Army Institute of Pathology is given by 5 year periods in Table 1. The apparent relative increase in the incidence of adrenocortical contraction has been pointed out many times in this country (Wells, Humphreys and Work, 1937; Duff and Bernstein, 1933) and in England (Hellier, 1930; Susman, 1930; Barnard, 1930), but it is

not known whether the condition is really more frequent or whether the incidence of adrenal tuberculosis is decreasing.

The 21 cases of adrenocortical contraction (Table 2) include 17 in which there was bilateral contraction, 2 in which there was unilateral contraction and the opposite adrenal was not found at autopsy and 2 in which neither adrenal was found. Since it is well known that a de-

TABLE 1. RELATIVE INCIDENCE OF ADRENAL TUBERCULOSIS AND CONTRACTION IN 52 CASES OF ADDISON'S DISEASE IN THE FILES OF THE ARMY INSTITUTE OF PATHOLOGY

Period	No. of cases with Adrenal Tuberculosis	No. of cases with Adrenal Contraction
1915–20	2	1
1921–25	3	0
1926–30	7	3
1931–35	4	0
1936–40	5	2
1941–46	10	15
Total	31	21

formed, shrunken adrenal may be difficult to identify, inclusion of the 2 equivocal cases appears justified.

CLINICAL FEATURES

Significant clinical data in the 25 cases of the Institute series are listed in Table 2. All the patients were men; except for 1 Indian and 1 Negro, they were white.

Seven patients had known Addison's disease with all the typical clinical manifestations, and in 4 other instances this diagnosis had been considered. Seven patients received hormonal therapy. Some died during an infection or as the result of unusual strain, such as that occasioned by a trip away from the hospital. Others for whom the diagnosis of Addison's disease had been made or suspected died suddenly and unexpectedly before specific therapy was begun.

In 7 instances the clinical syndrome was so atypical and confusing and the course of the disease so short that the diagnosis of adrenal insufficiency was not entertained. Consequently, only 1 of the 5 patients who died within a day after hospitalization received hormonal therapy.

Nausea, anorexia, vomiting, diarrhea and abdominal pain are common symptoms of Addison's disease. Nevertheless, 2 patients had vomiting and diarrhea so extreme that they were believed to have been poisoned, and extensive toxicologic studies were carried out. Neuropsychiatric manifestations dominated the picture in 5 instances. Three patients were considered psychotic, 1 was thought to have suffered an intracranial hemorrhage and 1 was given the diagnosis of myasthenia gravis. One patient was operated on for supposed rup-

TABLE 2. CLINICAL DATA IN 25 CASES OF ADRENAL INSUFFICIENCY

Case no.	Patient's years	Clinical manifestations	Duration	Clinical diagnosis	Comment
		Bilateral Adrenocortical Contraction			
1	33	Gastrointestinal disturbances, hypotension, pigmentation, neuropsychiatric disturbances, weakness	6 months	Psychosis; malnutrition; Addison's disease (?)	Received hormonal therapy; episode of weakness following pneumonia 2 years before fatal illness
2	30	Anginal seizures	1 day	Coronary occlusion	Pericarditis
3	18	Gastrointestinal disturbances, hypotension, pigmentation, weakness	1 month	Addison's disease (?)	—
4	32	Gastrointestinal disturbances, hypotension, pigmentation, weakness, neuropsychiatric disturbances	1 month	Psychosis; Addison's disease (?)	—
5	23	Weakness, hypotension, loss of weight	2 weeks	Myasthenia gravis; Addison's disease (?)	—
6	41	Semicoma, hypotension	1 day	Psychosis	Previous collapse after forced march
7	22	Gastrointestinal disturbances, hypotension, pigmentation, weakness	2 months	Addison's disease	Treated with sodium chloride, quinine, plasmochin; pericarditis
8	25	Hypotension, pigmentation, weakness, foot drop	7 months	Addison's disease	Received hormonal therapy; relapse during infection of respiratory tract
9	22	Abdominal crisis, pigmentation, electrocardiographic changes	1 day	Perforated peptic ulcer; coronary occlusion	Laparotomy; pericarditis
10	24	Coma, convulsions	1 day	Acute gastroenteritis; poisoning (?)	—
11	27	Gastrointestinal disturbances, hypotension, pigmentation, weakness	7 weeks	Addison's disease	Received hormonal therapy; relapse while out of hospital
12	22	Hypotension, pigmentation, weakness, gastrointestinal disturbances	9 months	Addison's disease	Previous psychotic episode; received hormonal therapy; relapse during infection of respiratory tract
13	23	Gastrointestinal disturbances, hypotension	1 week	Gastritis; poisoning (?); heat stroke (?)	—
14	38	Gastrointestinal disturbances, hypotension, pigmentation, weakness	6 months	Addison's disease	—
15	45	No data	—	—	Pigmentation & alopecia at autopsy
16	35	No data	—	—	Pigmentation & emaciation at autopsy
17	—	No data	—	—	Pigmentation & emaciation at autopsy
		Unilateral Adrenocortical Contraction (Other Adrenal Not Found)			
18	20	Infection of respiratory tract, gastrointestinal disturbances, pigmentation, coma	1 day	Addison's disease	Previous poisoning by carbon monoxide; received hormonal therapy
19	20	Gastrointestinal disturbances, weakness, hypotension	2 weeks	—	Previous thyrotoxicosis; thyroidectomy; received thyroid
		Neither Adrenal Found			
20	45	Coma	4 days	Intracranial accident	—
21	24	Hypotension, weakness, gastrointestinal disturbances, pigmentation	1 week	Addison's disease	Received hormonal therapy
		Acute Adrenal Lesions			
22	25	Gastrointestinal disturbances, hypotension, pigmentation, weakness, loss of weight	2 weeks	—	Previous malaria, dysentery; received hormonal therapy
23	42	Gastrointestinal disturbances, hypotension, weakness, emaciation	3 months	Malnutrition; psychosis	Purpura; nutritional anemia (?)
24	44	Gastrointestinal disturbances, hypotension, uremia	5 days	Nephritis	—
25	22	Gastrointestinal disturbances	1 day	Poisoning (?)	Had been drinking heavily for 2 weeks; found dead in bed

tured peptic ulcer. Two patients were believed to have had acute coronary occlusion; both had fibrinous pericarditis as well as adrenocortical contraction. It is worth pointing out that a third patient, with clinically typical Addison's disease, had fibrinous pericarditis at autopsy.

The diagnosis of Addison's disease in some of the cases with atypi-

1

2

3

4
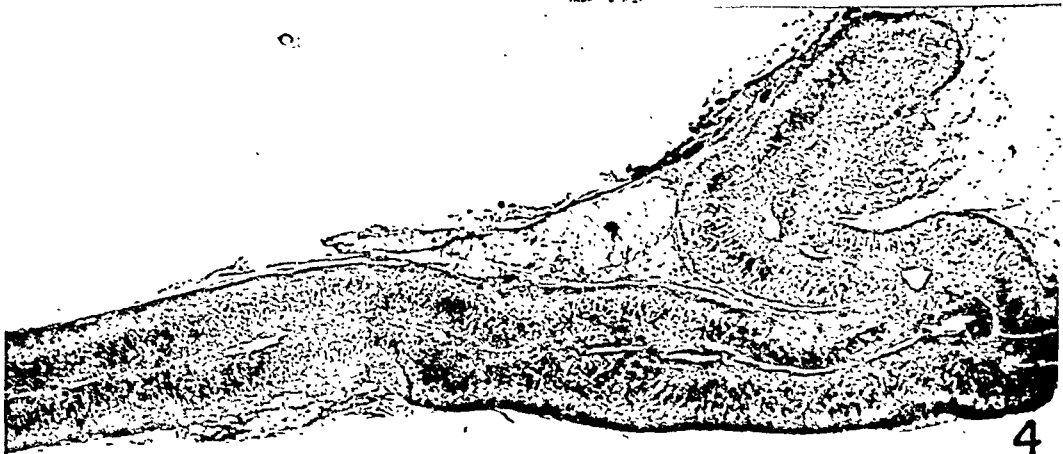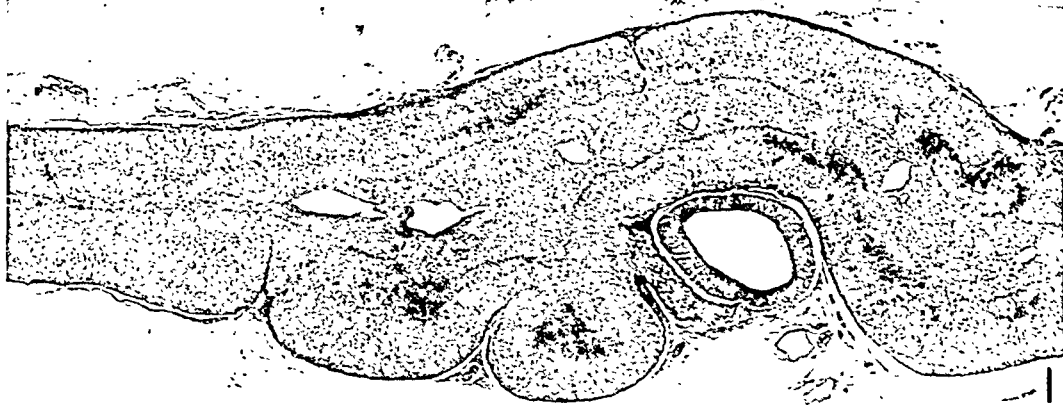
cal clinical pictures might be questioned. However, since adrenocortical contraction was the only significant lesion disclosed by autopsy, it seemed reasonable to ascribe death to adrenal insufficiency despite the absence of the full blown clinical syndrome. Furthermore, in some instances pigmentation, which had not been noted during life, came to light at autopsy; and in still others it was recalled in retrospect, after autopsy, that the patient had shown a remarkable response to saline infusions.

MORPHOLOGIC FEATURES

The adrenals were usually small and misshapen. The combined weights of the two glands were not infrequently as low as 2.5 Gm. The microscopic appearance of the contracted adrenals varied. In some cases simple collapse and shrinkage of the cortical parenchyma had taken place, so that the original outline of the gland was preserved (Fig. 2). In such cases the adrenal was made up almost entirely of medullary tissue, with only remnants of cortex (Fig. 5). The peripheral portions of the glands, where no medulla was present, were reduced to mere thin bands (Fig. 6). In the collapsed cortical portions the preexisting argyrophilic recticular framework was condensed (Fig. 7), but there was no productive or cicatricial fibrosis.

Residual groups of cortical cells persisted in places, and nodular proliferation (Fig. 3) sometimes obscured the over-all shrinkage. In 1 case hyperplastic nodules extended far into the periglandular fat (Figs. 3 & 9) and simulated a neoplastic process. The extracapsular cortical tissue in this case (Fig. 9) had to be differentiated from the "glandular" adipose tissue (Fig. 10) to which the periadrenal and other fatty deposits had been transformed in many other cases.[1] The residual cells and those in the proliferated nodules had a bizarre appearance. They were sometimes huge, with a cytoplasm deeply pigmented with brown granules and large, hyperchromatic nuclei (Fig. 8). The cellular groupings did not conform to the regular columnar and zonal arrangement of normal adrenal cortex (Fig. 12).

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[1] *Note:* After this study was completed another example of extracapsular proliferation of cortical cells was called to my attention (Zamcheck). In that case the patient died of diphtheria but had adrenocortical contraction and other evidence of latent Addison's disease.


Fig. 1. Photomicrograph of a normal adrenal gland, taken at the same magnification as Figs. 2, 3 and 4, ×10.

Fig. 2. Contracted adrenal; the medulla makes up almost all of the parenchyma. Compare with the size of the normal adrenal in Fig. 1. The cortex has been reduced to a thin shell barely thicker than the capsule. Case 12. ×10.

Fig. 3. Nodular hyperplasia of cortical tissue obscuring the underlying loss of parenchyma in an adrenal gland which has undergone marked contraction. Proliferation of auxiliary cortical tissue in the periglandular fat has also taken place. Case 10. ×10.

Fig. 4. Atrophy of the adrenal cortex secondary to a hypothalamic-hypophyseal lesion. The uniform shrinkage without distortion or contraction differs completely from the destructive "atrophy" of adrenocortical contraction. ×10.

CX

M

5

6

CP

CP

7
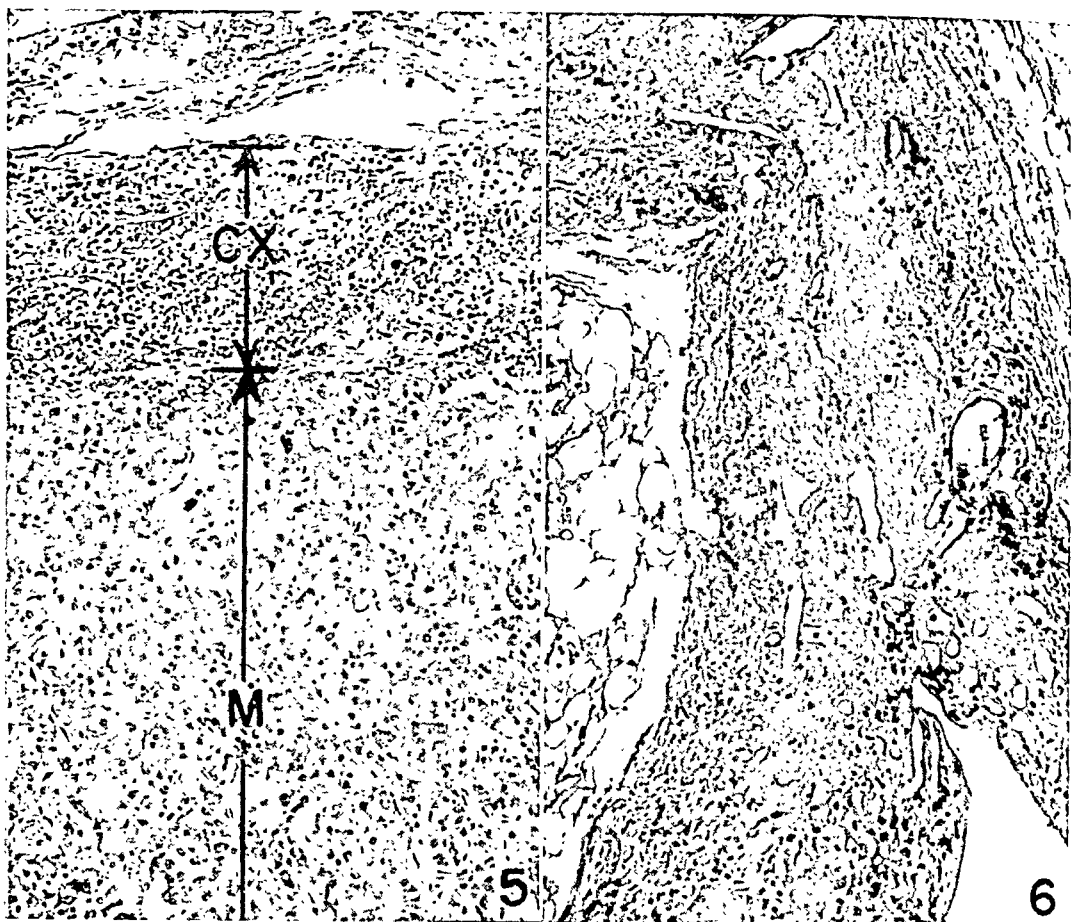
Stains which brought out the argyrophilic reticulum clearly showed the unoriented and confused architectural patterns (Fig. 11).

Although there was no loss of medullary tissue, the medulla was often heavily infiltrated with small dark round cells. In 1 adrenal such cells formed clear-cut lymph follicles (Fig. 13).

<div align="center">MORPHOGENESIS</div>

Most workers do not share Brenner's (1928) view that hyperplasia precedes rather than follows the cortical damage. The reasonable concept that necrosis of cortical cells precedes contraction is widely held. Duff and Bernstein (1933) and Susman (1930) pointed out that localized adrenal lesions resembling those diffusely distributed in adrenocortical contraction are seen from time to time at autopsy, and they suggested that summation of such damage could result in the full blown picture of adrenocortical contraction. It is true that focal necrosis and other degenerative lesions of the adrenal cortex are commonly encountered in a variety of conditions (Zamcheck), but such lesions probably could not result in the extensive, catastrophic, parenchymal destruction which is assumed to precede the ultimate contraction. Wells, Humphreys and Work (1937) pointed out that only massive necrosis could account for the final anatomic picture and expressed the opinion that an accumulation of minor atrophic lesions (Susman, 1936) could scarcely be adequate to produce it. The massive hemorrhage and necrosis of the adrenal which characterize the Waterhouse-Friderichsen syndrome could conceivably culminate in contraction, but there is no evidence to relate the two conditions. Signs of old hemorrhage were not seen in any cases of the Institute series. A history of episodes of weakness associated with infections suggested to Kiefer (1927) that toxic infectious processes might lead to adrenal contraction. In case 1 of the present series there was profound weakness following pneumonia.

Massive cortical degeneration, necrosis and inflammation, which might represent the hypothetical first stage in the development of adrenocortical contraction, were seen in 4 cases studied at the Institute. The clinical histories in these 4 cases are similar to those in the other cases of the series (Table 2). The adrenal changes seen microscopically in these 4 cases were as follows.

Case 22: Although no completely normal cortex remained, the

FIG. 5. The medulla (M) is preserved, but the cortex (CX) is reduced to a thin layer. Case 11. × 125.

FIG. 6. Extreme contraction of adrenal cortex. This section is taken from the peripheral portion, where medullary tissue is normally absent. A small amount of condensed interstitial tissue is all that lies between the apposed capsules. Case 8. ×125.

FIG. 7. Collapsed portion of adrenal cortex, similar to that illustrated in Fig. 6; stained for argyrophil reticulum (Wilder). Between the opposite capsules (CP) only a condensed network of the pre-existing reticular and sinusoidal structure remains. Case 12. ×240.
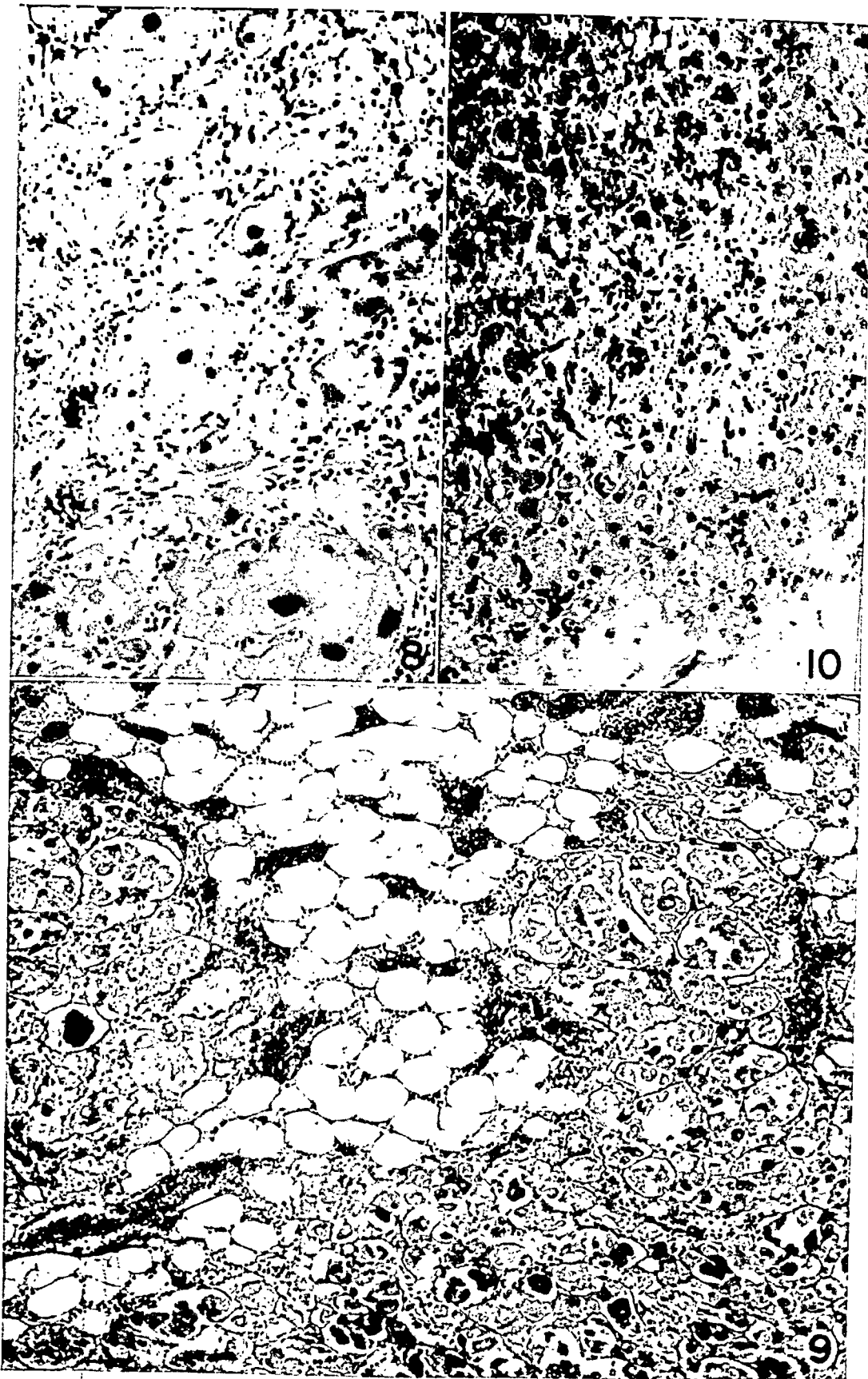
medulla was intact. In most places the reticular fibers had disappeared or were damaged; focally, the original pattern was preserved. Most of the cortex consisted of blood-filled sinusoids arranged in the usual pattern, running perpendicular to the capsule. Between the sinuses were a few necrotic cortical elements, cell "ghosts," extravasated red cells and lymphocytes (Fig. 14). In a few places, almost invariably in the outer portion of the cortex, viable parenchymal elements were seen. Although some were arranged in a way which suggested the previous columnar pattern, others formed little nodular aggregates. Occasional collections of lymphocytes studded the degenerated cortex, and there was diffuse lymphocytic infiltration about the persisting cell clusters.

Case 23: The cortex showed a band-like zone of degenerated adrenal tissue which traversed the entire gland. The medulla was unaffected. The zona glomerulosa was for the most part spared, but the zona fasciculata showed degeneration or disappearance of the cellular elements, complete in some places and partial in others. In most places cell "ghosts," in the form of acidophilic vacuolated coagulated masses, were evident. The nuclei when present were pyknotic or crenated. The appearance was not unlike that of zonal necrosis of the liver (Fig. 15). The reticular framework was practically unaltered. Few reactive leukocytes were noted.

Case 24: The entire cortex was made up of columns of degenerating and hyperplastic parenchymal cells. Vacuolization of individual cells or tubular and glandular transformation of the entire cord (Fig. 16) characterized most of the tissue. Some cells had acidophilic, hyaline masses or inclusions in the cytoplasm. Precipitated protein formed lacy networks in the pseudoglandular cavities. Between the abnormally widely spaced cortical columns the condensed stroma was prominent, and the tissue spaces contained edema fluid, red cells and precipitated protein. Where "regenerative" features predominated, the "irritated"-appearing cells had ample granular cytoplasm and active looking "owl's eye" nuclei containing large nucleoli. The cells were grouped in cords several layers thick, and in some areas solid sheets were formed. Nodularity was not noted. The reticular fibers were destroyed in many places, but where they persisted the altered architecture of the cortical columns disturbed their patterns.

Case 25: The fascicular zone of the entire cortex exhibited disinte-

FIG. 8. Giant and distorted cortical elements are often present in contracted adrenal glands. Many such cells contain dark brown pigment. Case 11. ×200.

FIG. 9. In this instance of adrenocortical contraction there was marked secondary nodular hyperplasia; accessory cortical tissue extended far into the periadrenal fat. Extracapsular cortical cells are sometimes difficult to differentiate from fat cells which have undergone glandular or embryonal transformation (See Fig. 10). Case 10. ×125.

FIG. 10. Glandular fat in the adipose tissue surrounding a contracted adrenal. Such altered fat cells may be difficult to differentiate from adrenocortical elements. Case 11. ×210.
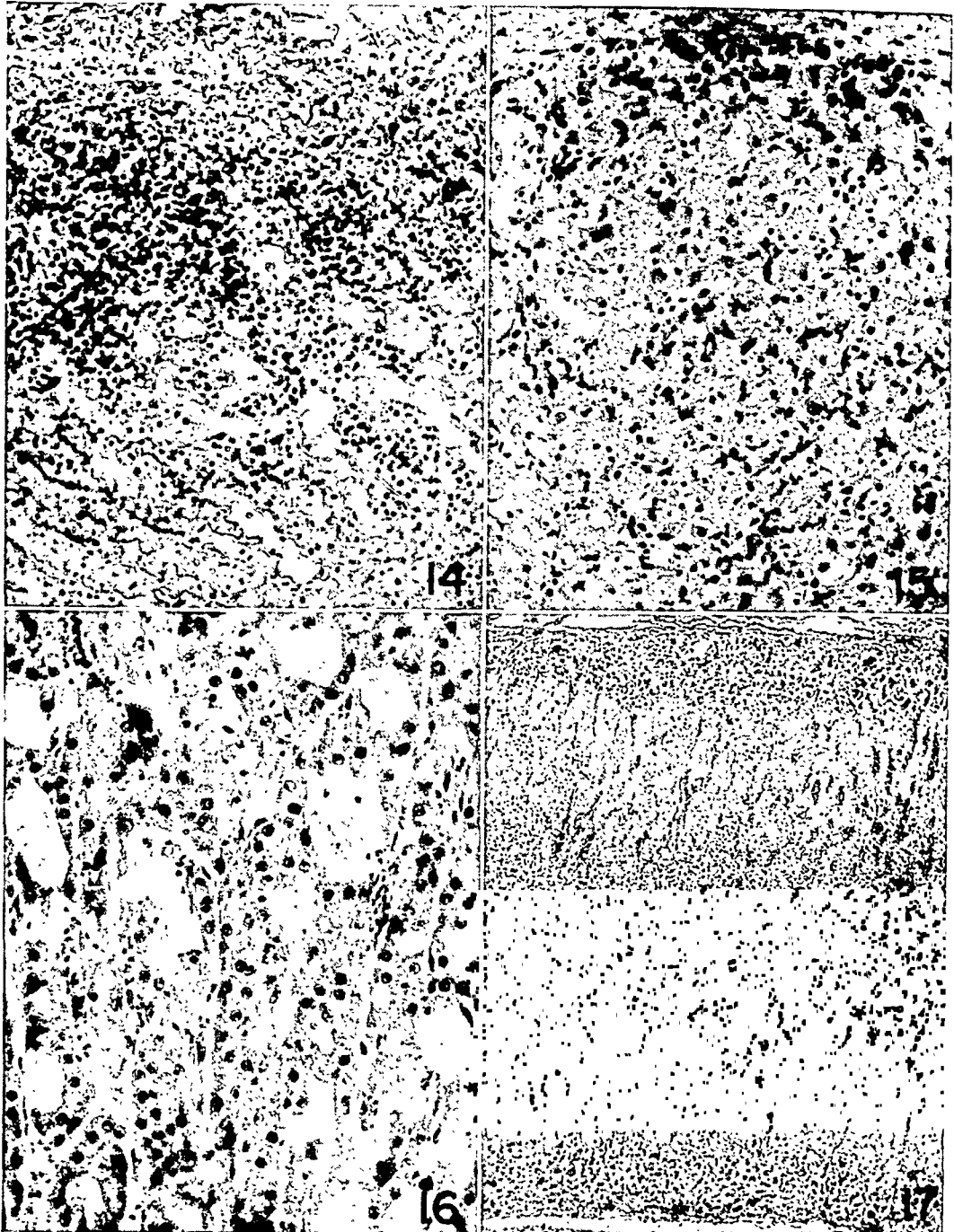
Fig. 14. Acute postnecrotic "collapse" of adrenal parenchyma. A few cortical cells remain immediately beneath the capsule. Most of the cortical elements have disappeared, leaving dilated sinusoids, reticular fibers and groups of round cells. Case 22. ×235.

Fig. 15. Acute zonal cortical necrosis of adrenal. A thin rim of relatively unaltered cortical cells remains immediately beneath the capsule. Most of the parenchymal elements have undergone coagulation necrosis and/or lysis. There are a few leukocytes. Case 23. ×160.

Fig. 16. Acute universal "glandular" degeneration of adrenal cortex. Cavities have

in which there was tuberculosis of one adrenal and atrophy of the other. However no one has suggested that the morphologic features of adrenocortical contraction resemble those of a tuberculous lesion.

It has recently been suggested (Rogoff, 1944), because ischemic damage to the adrenal which resulted in functional insufficiency has been produced experimentally in animals, that vascular occlusion could be held responsible for adrenal "atrophy" in Addison's disease. It is reasonable to assume, as did Rogoff (1936), that the Addison's disease which developed in a patient who had been subjected to an operation in the vicinity of the adrenal probably resulted from compromising of the vascular supply of the gland. There is no doubt that vascular occlusion can cause significant lesions in the adrenal and may even result in Addison's disease. For example, the Institute's files contain material from a patient in whom adrenal insufficiency developed after thrombosis of the adrenal vessels. The lesions were typical infarcts (Fig. 19). Another patient, who did not have Addison's disease, died 3 weeks after nephrectomy, with lesions in the adrenal. The segmental involvement, cicatricial fibrosis, obliteration of vessels and frank infarction which were present (Figs. 21, 22 & 23) bear no resemblance to the features of the usual instance of adrenocortical contraction but are strikingly similar to those of the lesions produced experimentally by vascular occlusion (Rogoff, 1944). Rogoff's view that a chronic inflammatory process might cause "cytotoxic atrophy" through vascular occlusion cannot be accepted because of the striking differences between ischemic lesions of the adrenal and those of adrenocortical contraction.

Wells, Humphreys and Work (1937) and, later, Tomlinson and Cameron (1938) reported cases in which it appeared that germanin (Bayer 205) might have been responsible for adrenal necrosis and contraction and Addison's disease. Since the drug was administered as a therapeutic agent to patients with pemphigus, the possibility must be considered that the adrenal lesions were associated with pemphigus rather than with germanin. However, the lesions of pemphigus described by Goldzieher (1945; 1946) and Guimaraes and Vieira (1945) were not those of adrenocortical contraction. Similarly, study of the adrenals in the Institute's collection of 20 cases of various types of pemphigus revealed only the nonspecific regressive and degenerative changes to be expected in a severe illness or in association with a widespread vesiculating dermatosis. Nothing approaching the picture of contraction was seen. Humphreys and Donaldson (1941) were able to produce adrenal damage by administration of germanin

formed in the centers of many of the cords and the remaining cells are hyperplastic. Case 24. ×160.

Fig. 17. Acute cortical necrosis and "adrenalitis." The inner two thirds of the cortex is necrotic and infiltrated with leukocytes. Dilated sinusoids are prominent. Case 25. ×55.
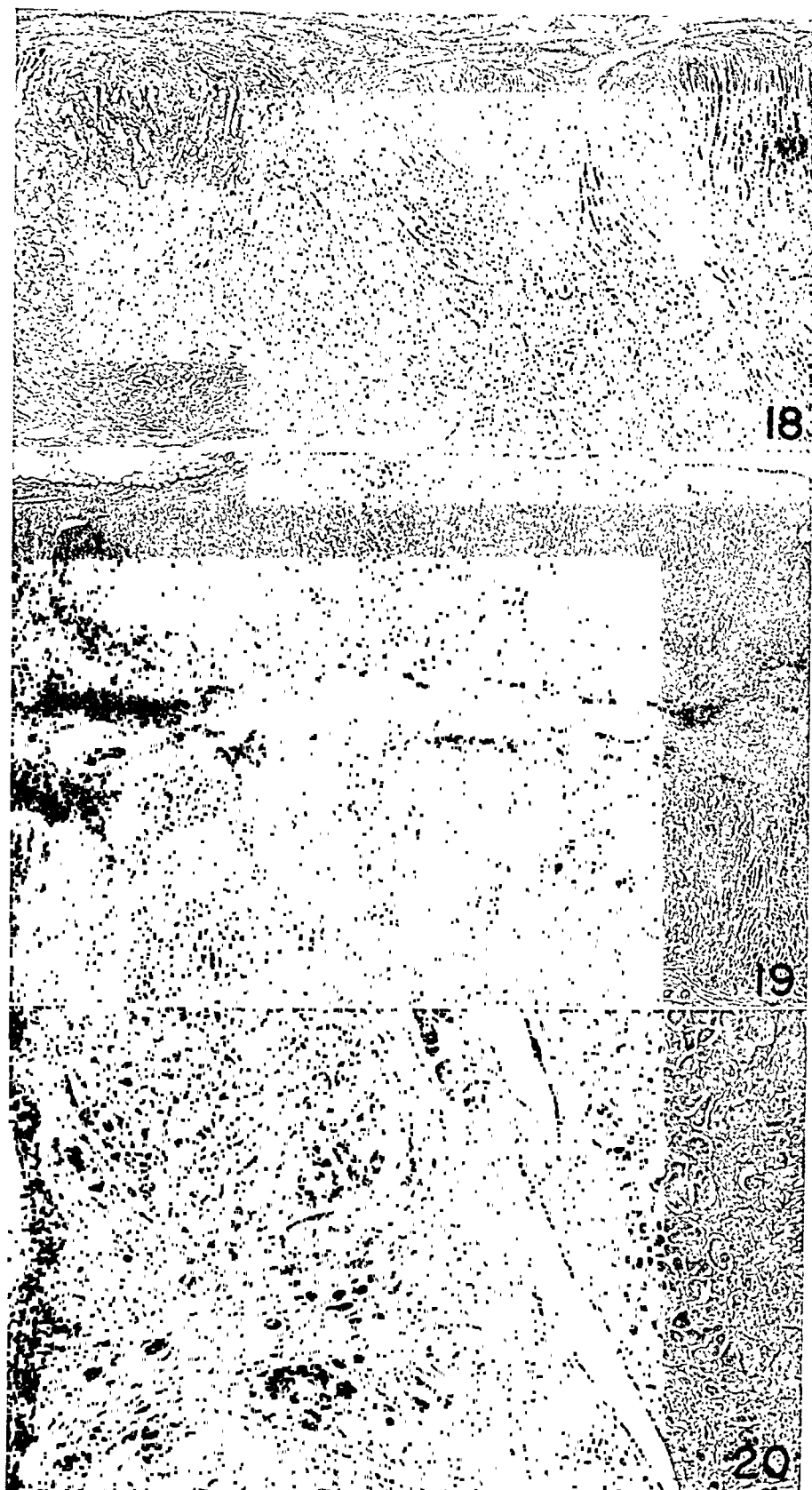
to guinea pigs, but Wells, Humphreys and Work (1937) stated that it is improbable that a drug used as rarely as germanin could have been responsible for many instances of adrenocortical contraction. They suggested that clinicians should be on the alert for adrenal effects from other therapeutic chemicals in more common use than germanin. They also pointed out that although germanin was extensively used in the treatment of trypanosomiasis (African sleeping sickness), nothing in the literature (MacLean and Fairbairn 1932; MacLean, 1928) indicated that with such therapy trypanosomiasis was complicated by adrenal lesions. However, the clinical picture of adrenal insufficiency might be obscured in a Negro patient dying of sleeping sickness (Wells, Humphreys and Work 1937), and local conditions might interfere with the performance of complete autopsies.

One patient in this series had a previous operation for thyrotoxicosis, but no evidence has been brought forth that endocrine factors bear any etiologic relation to adrenocortical contraction. The atrophic adrenal glands seen in association with damage to pituitaries or hypothalamic lesions (Fig. 4) might at times be confused with contracted adrenals, but the pattern of uniform atrophy without distortion or parenchymal loss is usually easily distinguished from that of adrenocortical contraction (Crooke and Russell 1935). I have, however, seen an adrenal lesion, probably endocrine in origin, which was almost indistinguishable from that of adrenocortical contraction; the patient, a man of 53, had been castrated and treated with estrogens (a-estradiol diproprionate and diethylstilbestrol), for carcinoma of the prostrate. Clinical evidence of adrenal insufficiency was not noted. Although the adrenals were not small there were irregular areas of cortical collapse and nodular proliferation (Fig. 20).

The observation of intranuclear inclusions in a contracted adrenal from a patient with Addison's disease suggested to Weiner (1936) "another interpretation." There is no evidence to support his implication that an infectious process might be responsible for the adrenal lesions, although the lesions of the liver in epidemic hepatitis (Lucké, 1944), which is known to be caused by a virus, are comparable to the adrenal changes in adrenocortical contraction.

COMMENT

There is no doubt that many features of adrenocortical contraction duplicate those of the lesions of certain diseases of the liver. Dis-

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Fig. 18. Syphilis of the adrenal in a patient who died of Addison's disease. The enlarged gland shows proliferation of both reticulum and collagen, with sclerotic destruction of the parenchyma. The pattern is entirely different from that of adrenocortical contraction (Wilder stain). × 15.

Fig. 19. Infarction of the adrenal. Although some cortical islands remain the patient had adrenal insufficiency. ×15.

Fig. 20. Adrenal lesions in a castrated man who had been treated with estrogens for carcinoma of the prostate. There are shrinkage of parenchyma and prolliferation of atypical cortical elements (Wilder stain). ×70.

the proper vascular connections and are not arranged in the normal lobular pattern. As in the thyroid, where hyperplasia may be associated with hypofunction, hyperplastic hypertrophic cells may be overworking, overstimulated cells which are not delivering a normal secretory end product to the blood stream.

The "glandular" or "embryonal" reversion of the adipose tissue, which was noted in the periadrenal tissues and in the fat in other locations, was striking. Such transformation of fat is not a specific process; it is encountered in a variety of emaciating diseases and is common in children. However, such marked alteration in a condition in which there is a deficient production of steroid hormones is of interest, since it is known that fat soluble hormones are at least stored in fat. Embryonal transformation of fat probably accounts for the pigmentation of adipose tissue described by Duffin (1943). Altered periadrenal fat must be differentiated from extracapsular cortical tissue in some instances.

The pericarditis present in 3 cases appears to be an unusual complication of Addison's disease. Terminal infection appears unlikely but not impossible as a cause, and an azotemic exudate cannot be altogether ruled out. In 2 cases the pericarditis had led to the diagnosis of coronary occlusion.

The danger of temporizing with the medical emergency of adrenal insufficiency is brought out by the clinical histories. Addison's disease should be seriously considered in the differential diagnosis of acute and subacute disturbances referable to the gastrointestinal tract, psychotic reactions and sudden collapse.

SUMMARY

Between December 1941 and December 1946 pathologic material from 15 patients who exhibited adrenocortical contraction at autopsy was received at the Army Institute of Pathology. During the same period specimens from only 10 patients with Addison's disease caused by tuberculosis were accessioned. Prior to World War II, only 6 cases of adrenocortical contraction had been studied at the Institute, as contrasted with 21 cases of tuberculous Addison's disease.

The clinical syndrome was in many instances so atypical and confusing that the diagnosis of adrenal insufficiency was not entertained, particularly when the disease ran a short, fulminating course. Acute gastro-enteritis, poisoning, ruptured peptic ulcer, coronary occlusion, psychosis, intracranial hemorrhage and myasthenia gravis were all simulated. The danger of temporizing with the medical emergency of adrenal insufficiency was underlined by the repeated occurrence of sudden collapse and death.

The morphologic picture in the 21 cases of full blown adrenocortical contraction ranged from that of pure destructive atrophy, or

collapse of the cortex to that in which the regeneration of cortical cells and nodules in atypical patterns overshadowed and masked the underlying atrophy. Although the early stage of "atrophy" is rarely encountered in material obtained at autopsy, the Institute files contain 4 examples of cortical degeneration, necrosis and inflammation so pronounced that one could conceive of their being the hypothetical precursors of adrenocortical contraction.

The lesions in adrenocortical contraction differ strikingly from those caused by vascular occlusion, syphilis and tuberculosis. They bear a strong resemblance to the lesions of necrotizing hepatic injury and its sequelae.

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PROGESTERONE AND DESOXYCORTICOSTERONE IN THE STEROID CONTROL OF THE GONADOTROPHIC FUNCTION OF THE HYPOPHYSIS

(Exemplified by the Behavior of the Intrasplenic Ovarian Graft in the Guinea Pig)

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LARGE blood follicles and cystic follicles appear in intrasplenic ovarian grafts in the castrated guinea pig (Lipschutz, 1946).[1] When the second ovary is left *in situ*, these blood follicles or cystic follicles do not occur. This gives full evidence that the phenomenon is due to the hypophysis being freed from the control by the ovary. Besides formation of blood follicles luteinization also becomes abnormal. There is partial luteinization of the wall of cystic hemorrhagic or non-hemorrhagic follicles, true lutein cysts can be found. Clusters of luteinized cells are scattered in the stroma. But corpora lutea are small at two months after transplantation; most of the corpora lutea do not attain the diameter of normal corpora lutea. From the third month on luteinization becomes overwhelming; large corpora lutea become numerous. At about ten months a luteomatous condition is attained though blood follicles continue to be found (Lipschutz *et al.*, 1946). The luteomatous degeneration of the ovary has been described by Biskind and Biskind (1944) in the rat and by various authorities in the mouse (Li and Gardner, 1947; Furth and Sobel, 1947). The luteomatous neoplastic condition in rats and mice, in any case in certain strains, is attained sooner than in the guinea pig; blood follicles have not been described by the authorities working with intrasplenic grafts in rats and mice. When comparing the ovarian condition in similar experiments in mice and guinea pigs it becomes evident that the evolution of the graft, including its neoplastic condition due to the rupture of the normal ovarian-hypophyseal relationship, differs with the species.[2] In the guinea pig the evolution of the luteomatous condition

is so slow that the intrasplenic graft offers a very good opportunity for reexamining, by administration of hormones, the steroid control of the gonadotrophic function of the hypophysis *in situ*, freed previously from gonadal control.[3] Certain difficulties may arise from adhesions between the spleen and the abdominal wall which allow a escape of an unaccountable quantity of estrogen into the general circulation. But in work with guinea pigs one is not much hampered by this accident when operating under aseptic conditions.

In the present paper we shall deal with experiments in which the action and interplay of α-estradiol, progesterone and desoxycorticosterone have been studied. Experiments with testosterone and other 3-keto-steroids are in progress.

PROCEDURE

Both ovaries were removed and one of the ovaries was grafted into the spleen. Two weeks later a pellet of the respective steroid, or steroids, was implanted subcutaneously. The animals were sacrificed two months after ovarian transplantation. The part of the spleen containing the ovary was fixed in Bouin's fluid to be cut two days later with the razor into slices about 1 mm. thick; presence of blood follicles, tertiary follicles and corpora lutea was noted. Representative slices were chosen in each case for microscopical examination. The corpora lutea were counted in a number of sections and the maximal number encountered in *one* section was taken as the "number of corpora lutea." Two diameters of the corpora lutea were measured in sections in the usual way; the "maximal corpus luteum diameter" (the average of the two maximal diameters measured) was thus determined for each animal. Similar measurements were made also for blood follicles and tertiary follicles. As to the quantitative statements about corpora lutea there were sometimes unsurmountable difficulties due to the irregularities of luteinization referred to above. Such cases were omitted.

EXPERIMENTS WITH α-ESTRADIOL

Results are summarized in Table 1.

When no steroid is added most grafts show blood follicles. Their incidence may vary from one series to another but their frequency is always overwhelming. In the present series *Ib* two thirds had blood follicles. Their diameter was in most cases greater than 1 mm., i.e. to the maximal diameter of a tertiary follicle we have found in a series of six normal adult guinea pigs; the diameter of blood follicles may attain as much as 2 mm. or more. The diameter of non-hemorrhagic tertiary follicles varied greatly; they were often cystic, attaining a diameter double that of the normal. With α-estradiol (group II) abnormal follicular growth was definitely counteracted, there being no blood-nor cystic-follicles. None of the steroids administered simultaneously with α-estradiol—progesterone (V), desoxycorticos-

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[3] This paper was already written when the work of Jungk, Heller and Nelson (1947) and of Heller and Jungk (1947) on the rat came to our knowledge.

terone acetate (VII)—were able to counteract this controlling action of the estrogen.

Another highly interesting aspect in our work was the considerable increase in luteinization under the influence of estrogen. Though, as already explained, corpora lutea are present and sometimes even numerous in the intrasplenic graft of the castrated guinea pig at two months after transplantation, they only exceptionally attain at this time the diameter of a normal corpus luteum, which is about 1.5 mm. On the contrary, when estrogen is administered, corpora lutea can be

TABLE 1

Group	Steroid	Absorb. per day μg.	Number of animals				Percentage of animals		
			Total (n)	With blood foll.	With C.l. (n₁)	With large C.l.*	With blood foll.	With C.l.	With large C.l.**
Ia*	None	0	19	18	12	2	95	63	10
Ib	None	0	29	19	12	2	66	41	7
II	α-estrad.	28–65	12	0	12	10	0	100	80
III	progest.	91–160	17	10	8	3	59	65	18
IV	progest.	180–469	23	14	4	1	61	17	4
V	α-estrad. and progest.	34–114 / 269–400	14	0	1	0	0	7	0
VI	desoxycort. ac.	178–320	8	4	1	0	50	13	0
VII	estrad. and desoxycort. ac.	17–72 / 180–490	13	0	2	0	0	15	0

* Ia is from a former series (Lipschutz *et al.*, 1946); for comparison with Ib.
** Diameter of 1.4 mm.

found in all animals; at two months 80 per cent of the animals, as against about 10 per cent without estrogen, have corpora lutea of at least a normal if not a larger diameter.

The degree of luteinization may be conveniently expressed in an index multiplying the average number of corpora lutea by the cube of the average of the maximal diameters in animals of the group. The procedure is indeed an arbitrary one, and as shown in the last column of Table 2, the index may vary greatly in animals without steroids. But the increase of corpora lutea formation under the influence of estrogen is overwhelming, there being a great increase both in the number and in the diameter of corpora lutea.

These findings with α-estradiol are corroborative of what is known from former manifold studies in which very different methods have been used.

RESULTS WITH PROGESTERONE

It may be said beforehand that these results, as shall be shown in the last section of this paper, acquire especial interest in relation with the findings and concepts of Smith and Smith (1946) on the role

of progesterone in the dynamics of the ovarian-hypophyseal relationship.

The incidence of blood follicles and corpora lutea was only influenced insignificantly, if at all, by the administration of progesterone alone (group III, table 1). With large quantities of the steroid diminution of the incidence of corpora lutea (compare groups IV with I) is rendered probable by our results. But interference of progesterone became fully evident in those experiments in which progesterone was administered simultaneously with α-estradiol (compare group II and V).

Progesterone even when given in large quantities (many times

TABLE 2

Group	Steroid	Corpora lutea			Index of luteinization axb³+10
		Total	Average per animal* (a)	Largest diameter** (b) mm.	
Ia	None	28	1.45	0.96	13
Ib	None	21	0.72	1.06	9
II	α-estradiol	26	2.17	1.7	106
III	progest. small dos.	17	1.00	1.08	13
IV	progest. large dos.	6	0.26	1.16	4
V	α-estr. and progest.	1	0.07	0.4	<1
VI	desoxyc. ac.	1	0.13	0.76	<1
VII	α-estrad. and desoxyc. ac.	3	0.23	1.0	2

* Total number of corpora lutea divided by total number of animal (n in table 1).
** The sum of the largest diameters divided by the number of animals with corpora lutea (n_1 in table 2).

those of α-estradiol) does not counteract the controlling action of α-estradiol as to blood follicles. There were no blood follicles in group V. Neither were there cystic tertiary follicles. On the contrary, the luteinizing influence of the estrogen is definitely inhibited by progesterone (table 1). All animals had corpora lutea when α-estradiol was given; addition of progesterone caused a drop from 100 per cent to 7 per cent. Even more striking was the drop of incidence of *large* corpora lutea—from 80 to 0 per cent. The index of luteinization (table 2) dropped from 106 to less than 1.

EXPERIMENTS WITH DESOXYCORTICOSTERONE ACETATE.

This steroid was tried alone (group VI) or in combination with α-estradiol (group VII). Desoxycorticosterone acts similarly to progesterone. There was no significant influence on the incidence of blood follicles. Inhibition of corpora lutea formation in group VI was no less pronounced than with similar quantities of progesterone (group IV and VI). There was also a definite inhibition of the luteinizing

EXPLANATION OF FIGURES

Intrasplenic autoplastic grafts in castrated guinea pigs. 60 to 65 days after transplantation, and 39 to 49 days of action of different steroids.—A. *Progesterone.* Large hemorrhagic follicles as in animals without steroids. Cystic follicle. Distension of the spleen (CI.85).—B. *α-estradiol.* No hemorrhagic follicles. Corpora lutea always present. Tertiary follicles small. No distension of spleen (CI.26).—C. *α-estradiol and progesterone.* No hemorrhagic follicles. No corpora lutea. Tertiary follicles small. No distension of spleen (CI.94 and 97).—All figures augment. ×10.

faculty of α-estradiol without the controlling action of the latter as to follicular growth being interfered with (group II and VII).

DISCUSSION

The results obtained with reference to the interference of progesterone in ovarian events can be summarized in the following two

statements: (1) interference of progesterone is as fundamental for the maintenance of the ovarian cycle as is α-estradiol; (2) under certain quantitative conditions progesterone counteracts or antagonizes the luteinizing influence of α-estradiol. As to luteinization there is definite evidence not only of a differential but also of an antagonistic behavior of the two steroids: α-estradiol calls for luteinization, and progesterone holds luteinization in abeyance. This differential and antagonistic behavior of the two steroids is an experimentally established fact which is highly suggestive of the concept that the normal sequel of the follicular and luteic phases of the ovary are under the dependence of an interplay of the two steroids mentioned. It is now that the question arises about how and where this interplay takes place.

Three possibilities have to be taken into consideration: (a) an interplay of the two steroids *in the ovary itself;* (b) an interplay in certain phases of the *metabolism and excretion of the steroids;* (c) an *intrahypophyseal interplay* of the two steroids controling the timing as to the release of the follicle stimulating and luteinizing gonadotrophic hormones.

It cannot be denied that estrogen may stimulate follicular development by direct action on the ovary (Pencharz, 1940; Williams, 1940, 1944; Simpson *et al.*, 1941). But the changes induced by the direct action of estrogen on the ovary of the hypophysectomized rat are just the opposite of those we are dealing with here: follicular development in the hypophysectomized rat is enhanced by estrogen, whereas it is "controlled" or "limited" in the intrasplenic graft in the guinea pig by estrogen administration. Direct action of estrogen on the ovary cannot serve as an explanation for those profound ovarian events which are consequent to the administration of estrogen in experiments with intrasplenic ovarian grafts. More significant, from the point of view of the dynamics of the steroid control of the follicular events in the intrasplenic graft, are the older findings of Westman and Jacobsohn (1937) and of Robson (1937) in the hypophysectomized pseudopregnant rabbit in which the corpus luteum remained histologically normal when estrogen was administered. No data are available about a direct action of progesterone on the ovary.

The statements of Smith and Smith (see summary 1946) relative to the enhancement of metabolic conversion of estradiol by progesterone are very suggestive. But even when fully admitting this type of progesterone interference in our experiments this would not mean dropping definitely the concept of progesterone partaking in some way in the steroid control of the hypophyseal gonadotrophic function by direct action on the anterior lobe.

The concept that estrogen enhances the release of the luteinizing hormone would be in accord with our present results. This is also in full accord with a former statement of the senior writer that whereas the faculty of the anterior lobe of the male donor rat to induce lutein-

ization in an immature female recipient diminishes considerably when estrogen has been previously administered for a certain time to the donor there is no concomitant diminution of the faculty to induce estrus in the immature recipient and to elicit an increase of the uterine weight (Lipschutz, 1935). It is very likely that the estrogen-induced release of the luteinizer has been counteracted by progesterone in our present experiments with intrasplenic grafts. But as evidenced by the condition of the graft, release of the luteinizer is not the only action of estrogen. There is also the fact that production of blood follicles is controlled by estrogen. This may be interpreted in the sense that estrogen puts a brake on an uncontrolled release of the follicle stimulating hormone. Should interference of progesterone in the conversion of estrogen be the only mechanism in play, without any action of progesterone on the anterior lobe, one should suppose that controlling the release of F.S.H. and enhancing the release of L.H. take place at different quantitative levels of estrogenic action. One should then assume that with larger quantities of progesterone administered simultaneously with the estrogen it should be possible not only to counteract luteinization as in fig. C but also to revert the ovary to a condition as in fig. A. The quantities of progesterone used in our experiments and still insufficient to counteract *both* aspects of estrogenic action were indeed considerable compared with those of α-estradiol administered simultaneously (269 to 400 μg of progesterone daily for 45 days; (table 1). Experiments with smaller quantities of the estrogen and larger quantities of progesterone are needed to settle this question.

On the other hand an action of progesterone on the anterior lobe with inhibition of the release of L.H. has been stated in the rat by Astwood and Fevold (1939) with quantities of 0.1 to 1 mg. of progesterone daily. On the contrary, inhibition of the release of F.S.H. was found only exceptionally, even with quantities so great as 3 to 6 mg. of progesterone daily during five to six weeks (Cutuly, 1941).

The slight action of progesterone and desoxycorticosterone, in absence of any estrogen (see group IV and VI, table 1 and 2), if significant, would be in favor of the concept that progesterone may act under certain quantitative conditions, also directly on the anterior lobe. But the objection may be raised that the diminution of luteinization in the two groups (IV and VI) compared with groups Ia and Ib, was due to conversion—in IV and VI—of those small quantities of estrogen which may have escaped inactivation. The results of Smith (1944, 1945) with Westerfield's lactone, an oxydative product of estrone, whose action on the hypophysis cannot be counteracted by progesterone, do not favor either the concept of an action of progesterone on the anterior lobe.

Whether progesterone interferes in the events in the intrasplenic graft exclusively by enhancing conversion of α-estradiol, or by direct intrahypophyseal action, it seems now clear enough that the problem

of the steroid control of the gonadotrophic function of the anterior
lobe is very far from being exhausted by the oversimplified concept
that estrogen serves as a "brake"of this function; even with quanti-
ties certainly much greater than physiological ones such a "brake"—
in an absolute sense of the word—has not been established in our work.
It may be definitely stated, however, that in the steroid control of the
gonadotrophic function of the anterior lobe progesterone is certainly
as fundamental as is estrogen. The work of Smith and Smith and
so also our new findings make it easy to understand why there must
be in the body intrahepatic inactivation not only of estrogen but also
of progesterone (Dosne, 1944) though in a quantitatively limited or
controlled manner (Bruzzone and Cuevas, unpublished).

Our finding that desoxycorticosterone controls the condition of
the intrasplenic graft in the same direction as does progesterone also
deserves attention as it constitutes another example of the overlap-
ping of actions of progesterone and desoxycorticosterone. Quantita-
tive data are as yet not available. But the statement is of importance
that the quantities of desoxycroticosterone acetate used in our experi-
ments were not greater than those necessary for the survival of the
suprarrenalectomized guinea pig (Bruzzone *et. al.*, 1946) and they are
still unable to produce any disturbance of the ionemia in normal
guinea pigs (Alvarez and Fuenzalida, 1946).

SUMMARY

The intrasplenic ovarian graft in the castrated guinea pig has
been made use of for the study of the seroid control of the gonadotro-
phic function of the anterior lobe, *in situ*, freed from the control of
the endogenous ovarian steroids inactivated in their passage through
the liver.

Blood follicles and cystic follicles which are a most characteristic
feature of the intrasplenic ovarian graft in the castrated guinea pig
fail to appear when α-estradiol is administered.

On the contrary, α-estradiol greatly enhances production of cor-
pora lutea which, when no estrogen is administered, are scarce in
similar grafts in the castrated guinea pig at two months after trans-
plantation.

When progesterone is administered follicular development with
production of blood follicles and cystic follicles is not inhibited; lut-
einization is slightly inhibited.

When α-estradiol and progesterone are administered simultane-
ously there are no blood follicles and there is no luteinization, that is
progesterone counteracts the stimulative action of estrogen on lutei-
nization but not the estrogenic control of follicular growth.

The problem of the interplay of the two essential ovarian steroids
in the control of the hypophyseal gonadotrophic function is discussed.

Desoxycorticosterone acts similarly to progesterone.

ACKNOWLEDGMENTS

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THE INFLUENCE OF HYPOPHYSECTOMY, THYROID-ECTOMY, AND OF BOTH HYPOPHYSECTOMY AND THYROIDECTOMY UPON THE FAT CONTENT OF THE LIVER OF THE DOG[1]

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THE CONTROL exercised by pituitary and thyroid glands upon lipid metabolism has been the subject of earlier reports from this laboratory (Chaikoff *et al.*, 1936; Chaikoff *et al.*, 1941; Entenman *et al.*, 1942a and b). Hypophysectomy *per se* has but little effect on the levels of the blood lipids of the dog (Chaikoff *et al.*, 1936). Provided caloric intake is not restricted, thyroidectomy, on the other hand, produces changes in the concentration of blood lipids of this animal (Chaikoff *et al.*, 1941). Definite elevations in the levels of blood cholesterol esters were observed as early as 7 days after excision of the thyroid gland, and by the time 29 days had elapsed definite increases in total fatty acids were also found. Not all blood lipid constituents, however, responded to the same degree; phospholipids and free cholesterol seldom showed pronounced increases after thyroidectomy. It was later shown that the high levels of blood lipids produced by extirpation of the thyroid glands are dependent upon the nutritional state of the animal (Entenman *et al.*, 1942a). The rise in cholesterol esters and total fatty acids can be inhibited by fasting or by limitation of caloric intake.

In dogs deprived of both hypophysis and thyroid, the blood lipids may rise to extraordinarily high levels (Entenman *et al.*, 1942b). The abrupt and striking rise in blood lipids that occurs after removal of the second gland, as compared with the lack of response after hypophysectomy alone, suggested that thyroid deficiency is the primary stimulus to the blood-lipid rise of the doubly operated dogs. The more uniformly high blood-lipid levels in hypophysectomized-thyroidectomized dogs than in thyroidectomized dogs was ascribed to a depression of whatever accessory thyroid tissue may have remained after thyroidectomy.

It is shown in the present investigation that the control of these

glands over lipid metabolism is not limited to blood. In dogs deprived of the thyroid alone, fatty livers *may* be present, but in those deprived of both glands fatty livers are an invariable occurrence. The lipid changes in the livers in these animals thus parallel those found in the blood.

<div align="center">EXPERIMENTAL</div>

Hypophysectomized Dogs—Throughout the periods of observation each dog received twice daily, at 8:00 A.M. and at 4:00 P.M., 15 gm. of lean meat per kilo, 10 gm. of sucrose, 4 gm. of bone ash, and 1 gm. salt mixture.[2] Vitamin supplements were provided by the addition of 3 cc. Sardilene[2] and 5 cc. of Galen B.[2] After receiving this diet for 2–3 weeks the dogs were hypophysectomized by a method previously described (Dandy and Reichert, 1938). In the procedure used in this study no cautery was applied to the base of the brain. In most cases the appetites of the dogs were excellent after hypophysectomy.

Thyroidectomized Dogs—The procedure employed for excision of the thyroid glands has already been described (Chaikoff *et al.*, 1941). Before the operation dogs T2, T3, T5, T12, T13, and T14 were fed twice daily a diet of 15 gm. lean meat and 5 gm. of sucrose per kilo per day. In addition each dog received daily 2 gm. of the salt mixture, 5 gm. of bone ash, 3 cc. of Sardilene oil and 5 cc. of Galen B. The detailed dietary treatment of these dogs has also been described elsewhere (Entenman *et al.*, 1942a).

The dietary intake of dogs T6, T7, T16, T17, and T19 was kept constant throughout the experiment. Each dog received twice daily 15 gm. of lean meat per kilo, 10 gm. of sucrose and the vitamin and salt supplements described above.

Hypophysectomized-Thyroidectomized Dogs—For 2–3 weeks after admittance to the laboratory each dog (except HT15) was fed twice daily 15 gm. of lean meat per kilo, 1 gm. salt mixture and 2 gm. bone ash. HT3, HT6, HT8, HT10, and HT11 received 2.3 gm. sucrose per kilo, whereas HT18, HT21, and HT22 received 10 gm. sucrose per meal. HT15 was fed 150 gm. of whole ground fish (Balto dog food) and 50 gm. of sucrose per meal. Each dog also received 3 cc. of Sardilene oil and 5 cc. of Galen B in the morning meal. The dietary intake of all dogs except HT15 was constant throughout the experimental period.

After a suitable control period, each dog was subjected to complete excision of its pituitary gland. Four to 6 weeks later their thyroid glands were removed. In all cases one or two of the parathyroid glands were preserved with their blood supply intact. All other parathyroid tissues found were transplanted into neighboring muscle. For the first two weeks after the removal of the thyroid glands each dog was fed 5 gm. of calcium lactate and 100 cc. of whole milk per meal in addition to the other dietary constituents. In all dogs except HT11, appetite was lost after removal of the second gland. The exact caloric intake recorded for each dog was, however, maintained by forced feeding.

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[2] In all diets referred to here Cowgill's salt mixture was used (Cowgill, 1923). Each cc. of Sardilene contained not less than 100 A.O.A.C. chick units of vitamin D and 600 U.S.P. units of vitamin A. The vitamin content of Galen B has been previously recorded (Montgomery *et al.*, 1941).

At the end of the periods of observations listed in the tables the dogs were anesthetized with nembutal and the livers ground, sampled, and analyzed as described in a previous paper (Chaikoff and Kaplan, 1934). Tissues were also taken for histological examination.

Completeness of hypophysectomy was established in all dogs at necropsy.

RESULTS

Hypophysectomized (H) Dogs—It has been shown elsewhere that at early intervals after excision of the pituitary gland, namely 2.5–4

TABLE 1. EFFECT OF HYPOPHYSECTOMY ON PLASMA AND LIVER LIPIDS OF THE DOG

Dog no.	Body weight		Period after hypophys-ectomy	Plasma lipids						Liver	
	Initial	Final		Cholesterol			Phospho-lipids	Total fatty acids	Total lipids	Weight	Total fatty acids
				Total	Free	Ester					
	kg.	kg.	months	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	gms.	per cent
H25	7.2	9.7	24	114	52	62	275	475	589	220	2.2
H26	7.6	8.1	24	109	58	51	204	372	481	170	1.9
H27	7.2	7.8	24	73	29	44	192	284	357	152	4.9
H28	6.2	8.2	24	105	28	77	313	426	531	176	2.7
H29	6.4	9.1	24	134	34	100	246	518	652	196	3.0
H32	4.1	8.3	32	82	39	43	400	552	634	265	3.8
H33	5.5	6.7	32	81	37	44	318	499	580	212	3.2

TABLE 2. EFFECT OF THYROIDECTOMY ON LIVER LIPIDS OF THE DOG

Dog No.	Body weight			Period after thyroidec-tomy	Liver	
	Initial	At thy-roidectomy	Final		Weight	Total fatty acids
	kg.	kg.	kg.	days	gms.	per cent
T6	13.3	13.3	11.7	197	446	2.3
T3	10.5	11.1	10.4	237	260	6.4
T16	8.7	8.7	8.0	365	253	6.6
T17	8.5	9.2	9.4	365	187	7.8
T19	9.0	9.0	9.2	365	252	11.5
T13	10.5	10.7	9.6	370	250	8.2
T7	8.5	8.6	8.5	387	201	5.9
T12	9.0	9.3	12.5	460	417	21.7
T14	12.5	13.0	15.3	460	419	20.2
T2	7.7	7.9	12.7	799	432	21.5
T5	9.5	9.9	13.2	799	431	22.7

months, the fatty-acid content of the liver remains within normal levels (Chaikoff *et al.*, 1936). The values recorded in Table 1 leave no doubt that the absence of this gland for as long as 32 months (960 days) is also without effect upon the lipid content of the liver.

Thyroidectomized (T) Dogs—The fatty acid content of the livers of dogs was measured at various intervals from 197 to 800 days after thyroidectomy (table 2). In 2 dogs that were sacrificed at 197 and 237 days, the fatty-acid content of their livers was not above normal. Five dogs were sacrificed between 365 and 387 days after excision of

this gland; in only one of them (T19) was the fatty-acid content in excess of 10 per cent. By the time 460 days had elapsed, however, the livers of 2 dogs contained about 20 per cent fatty acids. This concentration of fat was also found in the livers of 2 dogs that were sacrificed 800 days after thyroidectomy.

Hypophysectomized-Thyroidectomized (HT) Dogs—Fatty livers were found in 8 of the 9 dogs subjected to removal of both glands (table 3). In 3 of them the fatty-acid content of the liver was in excess of 30 per cent. In one (HT15) the fatty-acid content reached the astonishing value of 52.5 per cent.

TABLE 3. EFFECT OF HYPOPHYSECTOMY-THYROIDECTOMY ON
THE LIVER LIPIDS OF THE DOG

Dog No.	Body weight				Period of observation		Liver	
	Initial	At hypo-physec-tomy	At thy-roidec-tomy	Final	After hypo-physec-tomy	After thy-roidec-tomy	Weight	Total fatty acids
	kg.	kg.	kg.	days	days	days	gms.	per cent
HT3	8.5	9.1	10.9	10.7	45	70	450	13.7
HT6	9.5	10.2	11.2	15.0	46	126	390	3.9
HT21	7.9	7.8	7.8	14.5	45	217	398	14.3
HT18	10.2	10.5	12.1	17.4	46	250	405	32.5
HT15	6.5	6.8	9.2	9.0	47	310	375	52.5
HT22	8.2	8.4	8.8	19.7	44	311	412	11.2
HT11	6.3	6.6	6.8	11.0	45	374	285	12.5
HT10	8.6	9.7	11.3	20.5	49	398	1455	30.5
HT8	8.0	8.8	10.3	16.5	47	419	989	16.2

The histological appearance of these livers has been described elsewhere (Chaikoff *et al.*, 1943). In addition to fattiness, an increased fibrous-tissue proliferation or frank cirrhosis was observed in many of the HT dogs.

DISCUSSION

In view of the well-known role of dietary factors in the prevention of fatty livers, it should be emphasized here that the thyroidectomized and thyroidectomized-hypophysectomized dogs were fed a diet high in protein. Each dog ingested daily approximately 300 gm. of lean meat. The development of fatty livers in these dogs. therefore, cannot be ascribed to their failure to ingest a diet adequate in lipotropic factors.

A marked gain in weight was observed in most of the HT dogs in which fatty livers were found. That this gain in weight, however, is not the cause of the fatty livers is borne out by the failure of the hypophysectomized dogs to develop fatty livers, though they too showed pronounced weight-gains during the period of observation. This view is reinforced by the finding (to be reported in the next paper) that choline prevented the accumulation of excessive amounts

Following a suitable control-period, the dogs were subjected to complete hypophysectomy (Dandy and Reichert, 1938). In the procedure used in this study no cautery was applied to the base of the brain. After a lapse of 34–60 days the dogs were thyroidectomized. One or two of the parathyroid glands were preserved with their blood supply intact. All other parathyroid tissue found was transplanted into neighboring muscle.

Following removal of both glands, the dogs were fed the diet described above *plus* one gram of choline chloride with each meal. Thus, throughout the entire period of observation after removal of both glands, each dog ingested, in addition to the regular diet, 2 gm. of choline chloride daily. Food not voluntarily eaten was force-fed.

To counteract the possible development of a parathyroid deficiency, each

TABLE 1. EFFECT OF CHOLINE ON LIVER FATTY ACIDS OF
HYPOPHYSECTOMIZED-THYROIDECTOMIZED DOGS

Dog No.	Body weight				Period of observation		Liver	
	Initial	At hypophysectomy	At thyroidectomy	Final	After hypophysectomy	After thyroidectomy*	Weight	Total fatty acids
	kg.	kg.	kg.	kg.	days	days	gms.	per cent
HT28	5.0	5.2	5.6	9.0	45	782	488	2.5
HT29	9.4	9.6	9.3	14.2	34	782	502	4.3
HT30	6.2	6.2	6.9	11.1	34	782	320	2.4
HT31	6.0	6.3	5.6	8.1	60	720	210	2.2
HT33	6.0	6.2	6.3	10.8	60	720	245	2.9

* After thyroidectomy each dog received 2 grams of choline chloride daily.

dog was also fed during the first 2 weeks after thyroidectomy 5 gm. of calcium lactate and 100 cc. of whole milk.

At the end of the periods of observation (table 1) liver and blood were removed and their lipid contents determined by methods previously described (Chaikoff and Kaplan, 1934). Free cholesterol was determined in an acetone solution from which phospholipids had been precipitated. Tissues were also taken for histological examination. The completeness of hypophysectomy was established in all dogs at necropsy by serial sections of the base of the brain.

RESULTS

The Fat Content of the Liver—It was shown in the preceding paper that, by the time 799 days had elapsed after excision of the thyroid glands alone, dogs had developed fatty livers (Entenman *et al.*, 1948). Thus in 2 dogs that were sacrificed at this time-interval after thyroidectomy, 21.5 and 22.7 per cent fatty acids were found in mixed samples of their livers. The earliest interval after thyroidectomy when a fatty liver was found was 460 days. Fatty livers appear much earlier in the dogs subjected to hypophysectomy as well as to thyroidectomy. Thus, by the time 217 days had elapsed after excision of the second gland (thyroid) their livers contained more than 10 per cent fatty acids. From 30 to 50 per cent fatty acids were found in the livers

of 3 of the doubly operated dogs that were sacrificed from 250 to 398 days after thyroidectomy.

In the present study, the doubly operated (HT) dogs were sacrificed at 2 intervals, i.e., 720 and 780 days after the second gland (thyroid) had been excised (table 1). Throughout this entire period each dog ingested daily 2 gm. of choline chloride in addition to the regular lean-meat diet. The highest value for the fatty-acid content of the livers of 5 dogs so treated was 4.3 per cent.

In view of the fatty livers observed in thyroidectomized and in hypophysectomized-thyroidectomized dogs not treated with choline, it must be concluded that the ingestion of *free* choline prevents the

TABLE 2. PLASMA LIPIDS OF HYPOPHYSECTOMIZED-
THYROIDECTOMIZED DOGS FED CHOLINE

Dog No.	After thy-roidectomy	Total cholesterol	Phospho-lipid	Total fatty acids	Total lipid
	days	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.	mg. per 100 cc.
HT28	782	181	825	2310	2491
HT29	782	192	1160	4540	4732
HT30	782	188	480	870	1058
HT31	720	438	575	835	1273
HT33	720	398	467	852	1319

accumulation of fat in the livers of the doubly operated dogs (table 1).

Blood Lipids—The plasma lipids of 5 HT dogs, which for 720 to 780 days after excision of the second gland received choline daily, are recorded in Table 2. The values for total lipids were well above 1000 mg. per cent. The concentrations of all constituents, namely total cholesterol, phospholipids, and total fatty-acids, were elevated. Thus the ingested choline, while it prevented completely the accumulation of extra fat in the liver, failed to prevent the rise in plasma lipids which we have previously recorded in HT dogs (Entenman *et al.*, 1942).

Body Weights—By the time they were sacrificed, all 5 of the choline-treated HT dogs showed considerable increases in their weights. This increase in weight was not unexpected, for, as already pointed out, the preoperative caloric intake was imposed upon dogs in which the metabolic rate had been reduced by excision of both glands (Entenman *et al.*, 1942). The prevention of fatty livers in the doubly operated dogs by the administered choline justifies the conclusion that weight gain *per se* was not responsible for the development of fatty livers in the HT dogs.

PATHOLOGICAL FINDINGS

In order to recognize the effects of choline upon the development of cirrhosis in the hypophysectomized-thyroidectomized dogs, it is necessary to compare the livers of 2 groups of dogs: those that ingested the 2 gm. of choline chloride daily for over 700 days and those that

received no choline. The livers of the latter will be described in detail elsewhere; hence the findings are merely summarized here for convenience of comparison.

a. *Hypophysectomized-Thyroidectomized Dogs Not Fed Choline*— The livers of 4 of the 9 doubly operated dogs (see preceding paper) were severely cirrhotic (fig. 4), while in the livers of 3 others fibrosis of varying extent and severity was present (fig. 2).

TABLE 3. PATHOLOGY OF CHOLINE-TREATED HYPOPHYSECTOMIZED-THYROIDECTOMIZED DOGS

Dog	Fat	"Plant-like" cells and hyalinization*	Hepatic fibrosis	Cirrhosis
HT28	0	4+†	2+‡	0
HT29	0	4+	0	0
HT30	0	2+	0	0
HT31	0	2+	0	0
HT33	0	2+	0	0

* See text for description and see figure 1.
† 2+ = About ¼ of lobules involved.
  4+ = About ¾ of lobules involved.
‡ 2+ = Fine cellular connective tissue in about ¼ of the lobules.

The most common fibrotic changes (previously described as precirrhosis) consisted of a thickening of the reticulum around the large fat cells near the central or sublobular branches of the hepatic vein (fig. 2). This reticular thickening was associated with an infiltration of round cells, polymorphs, and fibroblasts. In the later stages these precirrhotic changes extended farther into the lobule and along the course of the large radicles of the hepatic vein, and subsequently the fibrous bands around the hepatic radicles of neighboring lobules joined one another. This fibrosis, commencing around the radicles of the hepatic vein, when more advanced, results in distortion of the lobular architecture and incorporation of the portal tracts in the fibrous tissue (fig. 4). Ultimately, scar tissue obliterates the area originally enclosed by the joining of the fibrous tissue which commenced around the central veins of adjacent lobules.

In addition to this fibrosis initiated around the smaller divisions of the hepatic veins, another form of fibrosis commencing around the small portal tracts was detected in two livers. In the livers of two dogs

EXPLANATION OF PLATE I

FIG. 1, Dog HT30, fed choline. Note the very sharp cell walls and the granularity of the liver cell protoplasm ("plant-like" cells). The eosinophilic hyalinization of the liver cells associated with nuclear hyperchromasia is well shown. Round cell foci such as depicted here (top left corner) are common. Hematoxylin and Eosin ×225.

FIG. 2. Dog HT22, not fed choline. The pericentral fatty change is clearly shown together with the edema, cell infiltration, and early fibrosis (precirrhosis) around the central or sublobular vein. The small portal tract, below and to the right of the above mentioned lesion, is normal. Note also the early extension of the fibrosis along the fatty zones. Hematoxylin and Eosin ×70.
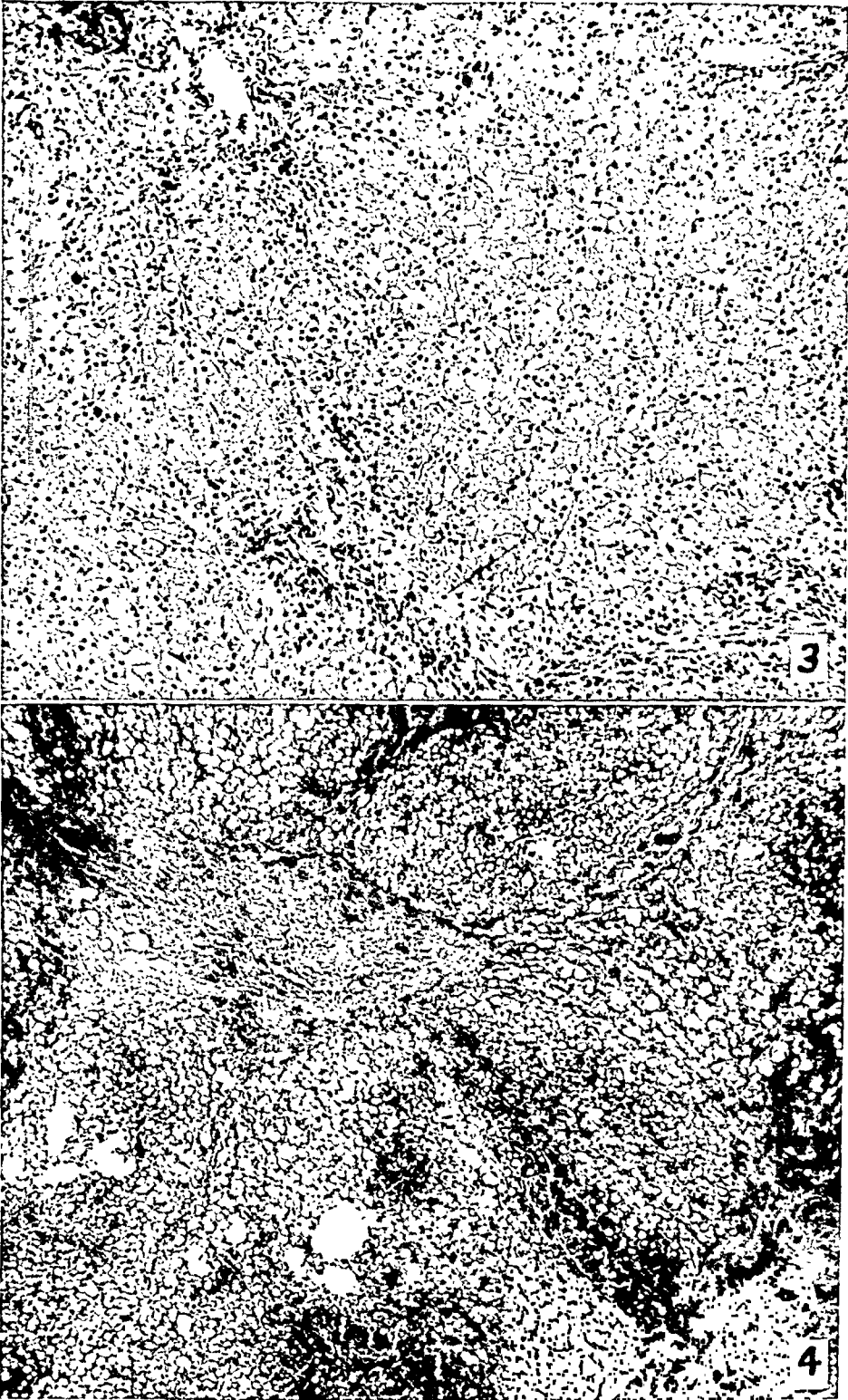
PLATE I

PLATE II

both periportal and perihepatic vein fibrosis were apparently progressing simultaneously.

b. *Choline-Treated Hypophysectomized-Thyroidectomized Dogs*—In only one of the 5 dogs treated with choline after ablation of both pituitary and thyroid glands, was any significant change observed in the liver. In this single animal changes regarded as indicative of early fibrosis were seen around many of the central veins, with complete absence of fat (fig. 3).

Neither fatty change nor evidence of fibrosis was detectable in the livers of any of the other dogs in this series. Apart from two peculiar changes in the cytoplasm of the liver cells and an unusual prominence of their cell walls, these livers could easily be taken for normal.

The two cytoplasmic changes in the liver cells to which we wish to draw attention are, firstly, a peculiar clumping of the chondriosomes which lie in a nonstaining cell sap and, secondly, an intense eosinophilia with hyalinization of scattered patches of the liver cells (fig. 1). In the former reaction the cell membranes of the affected liver cells are unusually thick and prominent ("plant-like" cells). These cannot be regarded as features of normal liver cells, but the significance of these changes is at present obscure.

However, despite these changes in the liver cells, it is clear from a comparison of figs. 1 and 3 with figs. 2 and 4 that the administration of choline has had a most remarkable effect in preventing the gross fatty changes so common in the untreated dogs deprived of both thyroid and pituitary glands. Moreover, the choline also diminished the incidence of cirrhosis and, in addition, in the one dog with pre-cirrhosis, its ingestion limited the extent and rate of progress of fibrosis.

DISCUSSION

A clue to the mechanism by which fatty livers develop in hypophysectomized-thyroidectomized dogs is provided by the observation that supplementation of their diets with 2 gm. daily of *free* choline chloride prevented this change. Each dog ingested daily from 200–300 gm. of lean meat; the lipotrope content of this amount of meat is more than enough to prevent fatty livers in normal dogs. The fact, therefore, that choline prevented fatty change in the liver means either 1) that the release of bound methionine and bound choline from dietary

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EXPLANATION OF PLATE II

FIG. 3. Dog HT28, fed choline. Note the cell infiltration and early fibrosis around both the small portal tract above and the centrolobular area below and to the right. This is the most severe degree of fibrosis encountered in the choline-treated dogs and is regarded as a very mild lesion. Hematoxylin and Eosin×110.

FIG. 4, Dog HT22, not fed choline. Note the severe fatty change, the obliteration of two lobules by fibrous tissue, and the early intralobular fibrosis between these two scars and towards the lower left. This severity of cirrhosis was encountered in several of the HT dogs. Comparison of this figure with fig. 2 above (both sections from different lobes of the same liver) reveals the extent of variation in reactivity which may occur in different lobes of a single liver. Hematoxylin and Eosin ×60.

constituents in the intestinal tract of the doubly operated dog is interfered with, or 2) the absorption of these substances is defective, or 3) that an interference in the utilization of lipotropic factors has occurred. As already shown, fatty livers do not develop in hypophysectomized dogs fed the stock diet but do occur after thyroidectomy, with or without hypophysectomy. This indicates that the effectiveness of the lipotropic constituents of a normal diet is, in part, dependent on the action of the thyroid hormone.

The action of choline observed here in preventing fatty infiltration and cirrhosis in the livers of dogs subjected to hypophysectomy and thyroidectomy should not be confused with a similar action of choline in rats fed a low-protein, low-choline diet (Blumberg and McCollum, 1941; Lillie et al., 1942; Engel, 1943; György, 1944). In the latter, the preventive action of choline upon both the fatty liver and cirrhosis was to be expected, since the rats were fed a diet deficient in lipotropic factors. This was not so for the choline effects reported here; choline completely prevented fatty livers and suppressed fibrosis in hypophysectomized-thyroidectomized dogs which throughout the experimental periods were fed a diet adequate in lipotropic factors as well as in other respects.

SUMMARY

The development of fatty livers previously reported in hypophysectomized-thyroidectomized dogs fed a high-protein diet can be prevented by the daily administration of 2 gm. of *free* choline chloride. Choline failed, however, to prevent the rise of the blood lipids in these dogs.

Choline also significantly suppressed or prevented the onset and development of hepatic fibrosis in these doubly operated dogs.

These results indicate that in the dog deprived of thyroid and pituitary glands there develops either 1) an interference in the availability or utilization of the lipotropic factors present in a meat-sucrose diet supplemented with vitamins and minerals or 2) an increased need for these substances.

The evidence obtained here indicates that the effectiveness of the lipotropic constituents of a normal diet is, in part, dependent on the action of the thyroid hormone.

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EFFECT OF THE DIETARY PROTEIN CONCEN-
TRATION UPON THE SECRETION OF
ADRENOCORTICOTROPHIN

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It has been previously reported (Dontigny, Hay, Prado and Selye, 1947) that animals chronically treated with large doses of crude anterior pituitary preparations become hypertensive if they are simultaneously fed a high (30%) protein diet. At that time attention was called to the fact that the hypertension was accompanied by a marked adrenal enlargement, while rats treated with the same pituitary preparation but receiving a low (15%) protein diet maintained a normal blood pressure and showed but slight adrenal stimulation. Subsequently, Prado, Dontigny and Selye (1947) showed that the concentration of protein in the diet does not affect the hypertension produced by desoxycorticosterone acetate overdosage. In order to give a plausible explanation to these observations, it was considered that the dietary protein could affect hormonal hypertension either by enhancing the response of the adrenal to adrenocorticotrophin or by increasing corticotrophin secretion by the anterior pituitary.

In order to gain further information concerning this point, we have studied the effect of the dietary protein concentration upon the responsiveness to stress of the hypophyseo-adrenal system. Two types of experiments have been performed: I, those based on the effect of injected corticotrophin upon the adrenal ascorbic acid; II, those concerning the compensatory adrenal hypertrophy (due to endogenous corticotrophin formation), as influenced by dietary protein.

I. ADRENAL ASCORBIC ACID

Methods and experiments. The suprarenal response to exogenous corticotrophin was estimated, using as a criterion, the decrease of the adrenal ascorbic acid concentration as described by Sayers, Sayers, Liang and Long (1945) and by Sayers and Sayers (1946).

This sensitive test has also been shown to be very useful in the study of endogenous corticotrophin activity of normal animals (Sayers and Sayers, 1947). In the present work, it has been used for both these purposes.

The adrenal ascorbic acid was determined by Carruthers' (1942) method. We slightly simplified it by estimating the ascorbic acid from a standard curve prepared from known amounts of vitamin C, instead of deriving it from the colorimetric readings before and after reduction of the standard 2, 6-dichlorophenolindophenol. The reliability of the method did not seem to be, in any way, impaired by this modification.

Four groups of male, adult, albino rats were fed the experimental diets (Table 1) during a period of three weeks. Groups. 1 and 3 received the 15%-

TABLE 1. COMPOSITION OF TWO BASIC SYNTHETIC DIETS
(parts per cent)

Diets	I	II
Casein	15	30
Corn Starch	78	63
Fat	1	1
Cod liver oil	1	1
Bulk	1	1
Mineral mixture	4	4
Supplements: Thiamine chloride	0.8 mg./100 gm. diet	
Riboflavin	0.8 mg./100 gm. diet	
Pyridoxine	0.8 mg./100 gm. diet	
Ca pantothenate	4.0 mg./100 gm. diet	
Nicotinic acid	1.0 mg./100 gm. diet	
Choline chloride	100.0 mg./100 gm. diet	
α-Tocopherol acetate	10.0 mg./rat/weekly	

casein-diet (Diet I), while groups 2 and 4 were fed a 30%-casein-ration (Diet II). At the end of the preparatory feeding period and after fasting the animals for 24 hours, groups 1 and 2 were exposed to a temperature of $4 \pm 1°C$. for one hour and the remaining two groups were kept at room temperature as controls. All animals were then killed with chloroform, and the ascorbic acid concentration of the adrenal glands was determined.

The same experiment was repeated, subjecting the animals to a greater degree of stress ($0 \pm 1°C$.).

Another experiment was then performed in order to ascertain the effect of the dietary protein concentration on the adrenal response to a given amount of exogenous corticotrophin. Two groups of adult, male, albino rats (70 to 85 gm.) were respectively fed diets I and II during three weeks at the end of which time they were hypophysectomized. Twenty four hours later, the right adrenal was removed under nembutal anesthesia, and immediately after, 10γ of corticotrophin was intravenously injected. One hour later the contralateral gland was also removed and the ascorbic acid concentration of each gland was separately determined. Care was taken to prepare the extract of each gland as it was extirpated. The cattle pituitary corticotrophin used was prepared by Fishman's (1947) method.

Results. Perusal of Table 2 reveals that exposure to cold caused a greater reduction of the adrenal ascorbic-acid concentration in ani-

mals receiving the high-protein-diet, than in rats fed the low-protein-diet. If the decrease in adrenal ascorbic acid is expressed as the percentage of the control value, the difference between each exposed group and its respective control is of the same order of magnitude in both experiments, 1 and 2, in spite of the admittedly, unexplained, low control values observed in the first experiment. These results indicate that the hypophyseo-adrenal system of animals receiving a

TABLE 2. INFLUENCE OF DIET UPON THE RESPONSE OF THE
HYPOPHYSEO-ADRENAL SYSTEM TO COLD

Group	Treatment	No. of rats	Diet	Adrenal ascorbic acid mg./100 gm. fresh tissue		
				Average ± S.E.	Decrease from control level	Decrease in percentage
1 3	Cold (exposed to 4±1° C. for 1 hr. before killing)	5 4	I II	247± 5.2 212±15.0	32±13.3 73±15.6	11.5 25.6
3 4	Unexposed controls	5 3	I II	279±12.2 285±29.2		
1 2	Cold (exposed to 0±1° C. for 1 hr. before killing)	7 6	I II	326± 5.5 286± 8.7	48±17.0 148±25.2	12.8 34.1
3 4	Unexposed controls	5 6	I II	374±16.0 434±23.8		
				Before corticotrophin injection (Left adrenal)	After corticotrophin injection (Right adrenal)	Decrease after injection	
1	Hypophysectomized 10 rats	6 11	I II	450±10.2 416±18.8	325±18.8 320±16.4	135±16.2 96±15.4	30 23

S.E. = Standard Error.

high-protein-diet gives a greater response to stress than that of animals fed a low protein diet.

These first two experiments, however, did not establish which gland of the hypophyseo-adrenal system was rendered more responsive to stress by the diet. It was in order to study the effect of the diet on the corticotrophin responsiveness of the adrenal itself that we injected the same amount of corticotrophin to hypophysectomized rats receiving one or the other of the two experimental diets. The results of experiment 3 (Table 2) show the decreases in adrenal ascorbic acid induced by the injection of 10γ of corticotrophin into two groups of animals pretreated with diets I and II. As the decreases observed were not significantly different (p = 0.1) it was concluded that the dietary protein concentration did not modify the adrenal response to exogenous corticotrophin.

II. COMPENSATORY HYPERTROPHY OF THE ADRENAL

Methods and experiments. The above results indicated that corticotrophin secretion is affected by the dietary protein concentration even in acute experiments, but we felt that a clearer effect might be observed in a long-term experiment. Considering that in the ascorbic acid test the actual period of

pituitary stimulation is limited to one hour, we thought that a study of the degree of adrenal compensatory hypertrophy, as affected by the dietary protein concentration, might furnish additional evidence of differences in pituitary activity.

In the first experiment, forty male albino rats (80–100 gm.) were divided into four groups: the first two groups received diet I (15% casein), and the last two, diet II (30% casein). After one week, the left adrenal was removed in all animals of groups 2 and 4, while groups 1 and 3 were kept as controls. Five animals of each group were killed on the fifth day after adrenalectomy

TABLE 3. EFFECT OF THE DIETARY PROTEIN CONCENTRATION ON THE
DEGREE OF ADRENAL COMPENSATORY HYPERTROPHY

(Average and Standard Errors)

Diets	Operation	No. rats	Autopsy days after adr-ect.	Final body weight gm.	Right adrenal weight mg.	Adrenal weight mg./100 gm. of body weight
I	Control	4	5 days	139 ±3.8	10.0 ±0.81	7.2
	Adr-ect.*	5		128 ±4.9	10.2 ±0.39	7.9
II	Control	4		145 ±5.8	10.9 ±0.76	7.5
	Adr-ect.	4		130 ±6.6	11.6 ±0.91	9.0
I	Control	11	10 days	155 ±3.9	9.9 ±0.36	6.4
	Adr-ect.	11		154 ±5.6	11.7 ±0.63	7.2
II	Control	10		178 ±4.1	9.8 ±0.27	5.6
	Adr-ect.	13		173 ±2.3	13.7 ±0.50	8.1
I	Control	10	15 days	179 ±3.9	9.7 ±0.23	5.4
	Adr-ect.	9		168 ±4.4	10.6 ±0.46	6.4
II	Control	8		187 ±3.9	9.2 ±0.32	4.9
	Adr-ect.	9		170 ±5.2**	13.3 ±0.45	7.8

* Adr-ect. = adrenalectomized.
** = Initial body weight proportionally smaller.

Significance of differences in average adrenal weight

5 days   10.2 ±0.39 ⎫
              11.6 ±0.99 ⎭ $p = 0.2$

10 days   11.7 ±0.63 ⎫
              13.7 ±0.50 ⎭ $p = 0.02$

15 days   10.6 ±0.46 ⎫
              13.3 ±0.45 ⎭ $p < 0.01$

and the other animals on the tenth day. The remaining adrenals in the operated groups and the right adrenals of the control groups were fixed in "Suza" for 24 hours, dissected and weighed on an analytical balance. This experiment was repeated twice: the second time all the animals were sacrificed 10 days after the unilateral adrenalectomy and in the third experiment, 15 days after the operation.

Results. As may be seen from Table 3, the degree of adrenal compensatory hypertrophy was markedly affected by the composition of the diet. The proliferation of the remaining adrenal gland was more rapid in the animals receiving diet II (30% casein) than in those fed

diet I (15% casein). This difference is significant ten or fifteen days following the operation whether calculated from the absolute or from the relative weights of the glands (mg. adrenal tissue/100 gm. body weight). The regeneration curves shown in Fig. 1 are obtained by plotting the percentage increases in relative adrenal weights against the experimental periods following unilateral adrenalectomy. It was not established whether the regeneration curve of the adrenal tissue of the animals receiving the 15%-casein-diet would eventually reach the curve of the 30%-protein-group or whether it would permanently remain at a lower level.

INFLUENCE OF THE DIETARY PROTEIN CONCENTRATION UPON THE REGENERATION-RATE OF ADRENAL TISSUE.

(Increases are expressed as % of the initial values in mg. adrenal tissue/100 gm. body weight. Time in days after unilateral adrenalectomy.)
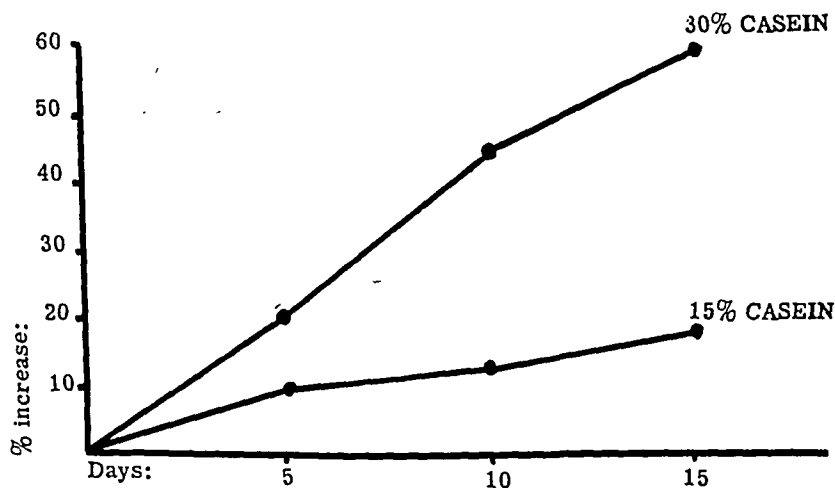
FIG. 1.

DISCUSSION

The evidence presented in this paper, derived both from Sayers' ascorbic acid test and from the regeneration of adreno-cortical tissue, seems to indicate that, under our experimental conditions, the dietary protein concentration affects the rate of corticotrophin secretion by the anterior pituitary. It is not possible to conclude with certainty from the available data whether the formation or merely the discharge of preformed hormone is altered by the diet. It would be difficult, however, to explain the more rapid regeneration of adrenal tissue induced by a higher protein diet in terms of increased discharge only, hence we are inclined to believe that the rate of secretion is increased. A third possibility, though quite hypothetical, is more difficult to eliminate, namely that the high-protein diet stimulates the elaboration by the hypophysis of corticotrophin-synergizing material.

No difference was found between the adrenals in the control groups receiving diet I (15% protein) and diet II (30% protein) respectively either in the ascorbic acid tests or in the compensatory hypertrophy experiments. This indicates that the diets used, do not—in themselves—induce a greater corticotrophin secretion. Only if the anterior pituitary is stimulated to increased corticotrophin production (by cold or by a partial corticoid insufficiency), does a difference appear between the animals receiving the high and the low-protein-rations.

The results obtained with the ascorbic acid test in hypophysectomized rats show that the adrenal cortex response to a known amount of corticotrophin is not modified by the diet. In this respect our observations confirm those of Ingle, Prestrud, Li and Evans (1947), regarding the effect of diet upon corticotrophin activity in intact rats. It is difficult to reconcile our results, however, with those of Benua and Howard (1945), who were unable to detect any effect of the dietary protein concentration upon the degree of adrenal compensatory hypertrophy. In the diets used by those authors, 66.8 to 89.3% of the total caloric value was derived from proteins; perhaps these concentrations were excessive and the diet as a whole too abnormal. It is noteworthy that the body growth of the unilaterally-adrenalectomized animals used in Benua and Howard's experiments, was significantly retarded; an effect which we did not observe with the dietary protein concentrations used in the course of this work.

SUMMARY AND CONCLUSIONS

Experiments on the rat indicate that the adrenal-cortical stimulation elicited by stress (e.g., cold) or by unilateral adrenalectomy is greater in animals kept on high (30%) than in those receiving low (15%) protein diets. Such a difference in the dietary protein concentration does not exert any significant effect upon the adrenal under basic conditions in normal animals, nor does it demonstrably affect the adrenal response of hypophysectomized rats to exogenous corticotrophin.

It is concluded that diets rich in protein do not sensitize the adrenal to corticotrophin, nor do they normally increase corticotrophin secretion by the pituitary. Under certain conditions, however, when adrenal growth is stimulated beyond normal, high-protein diets further augment corticotrophic response, either by increasing corticotrophin production or by eliciting the elaboration of some corticotrophin-synergizing hypophyseal principle.

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DISCUSSION

The increase in output of the glyconeogenetic factor after enucleation resembles that of the fat factor (1) in two ways: first, the maximum is far above normal, three times in this instance (if the glycogen deposition is in proportion to the hormone output); and second, a higher than normal level is maintained for several weeks following the operation. However, the significant difference as already indicated is the occurrence of widely separated maxima. The high point for the fat factor occurred at nine days, while that for the glyconeogenetic factor occurred at 29 days. There also appeared to
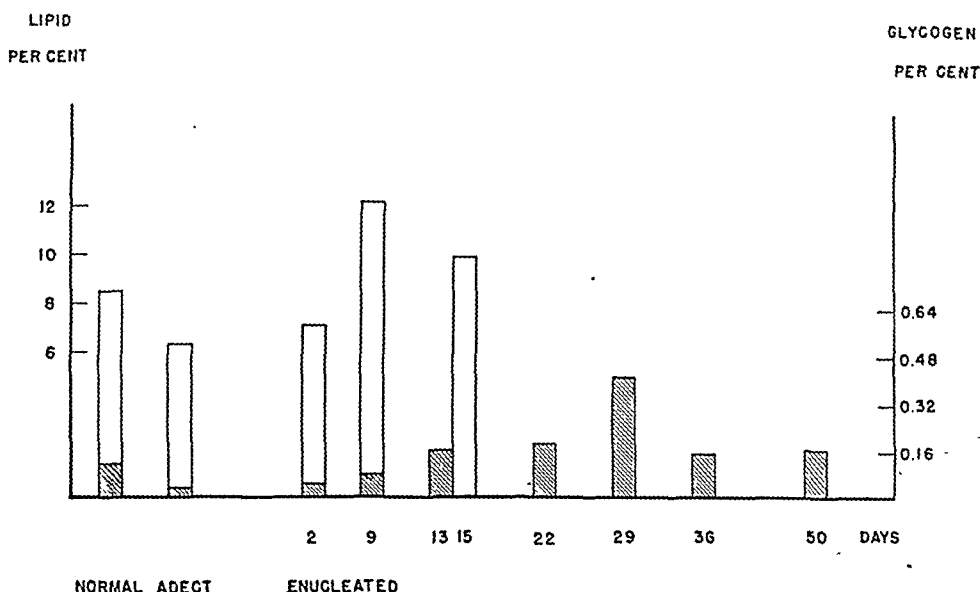
FIG. 2. Liver lipid and glycogen values after 24 hr. starvation, compared. Liver lipid values are taken from a previous publication (1).

be a more abrupt rise and fall to and from the maximum for the latter factor than for that for fat.

The obvious explanation of the increased hormone production after enucleation is that the sudden reduction of adrenal hormone output permits the pituitary to secrete more adrenocorticotrophic hormone which, in turn, stimulates the remaining cortical tissue to increased performance. As in the fat factor, it is difficult to understand why the increase in glyconeogenetic factor does not produce earlier inhibition of the pituitary than is apparent from the data.

The great range of values which occurs in all enucleated groups, and especially at the 29-day period, is noteworthy. We know from our own adrenal weight determinations after enucleation, in other studies not published, that the amount of regeneration varies greatly fron ome individual to another. Moreover, in some instances one adrenal may disappear entirely. Two explanations may be offered for the variation in glyconeogenetic response; either the pituitary fails to increase its adrenocorticotrophic hormone output as much in some animals as in others when adrenal tissue is reduced, or an unknown factor prevents the optimum regenerative response in some ani-

mals but not in others. Although a few cells can produce a very great increase in glyconeogenetic factor there is probably a limit to their output.

The occurrence of widely different maxima in point of time for the fat and carbohydrate factors indicates distinct hormones. Moreover it suggests two adrenocorticotrophic hormones.

Efforts are being made to separate these hormones from the respective glands.

SUMMARY

The amount of glycogen in starved mice was determined in normal and adrenalectomized animals and in animals whose adrenals had been previously enucleated.

Within two weeks after enucleation of both adrenals, the output of glyconeogenetic factor was much above normal. The maximum output, which was three times normal, was reached at about 29 days. Following this there was an abrupt fall to a level above normal which was maintained for at least three weeks.

It is pointed out that the maxima for the production of fat and glyconeogenetic factors occur at widely different times, which would indicate distinct hormones.

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THE USE OF THE MALE NORTH AMERICAN FROG (RANA PIPIENS) IN THE DIAGNOSIS OF PREGNANCY[1]

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WHILE it has been known for some time that the African clawed frog (Xenopus laevis) could be stimulated to mating by the injection of mammalian gonadotropins (Shapiro, 1936), this reactivity has only recently been utilized for the demonstration and titration of such hormones (Robbins, Parker and Bianco, 1947). It was found that the male frog reacted far more quickly than the female and furthermore was 64 times as sensitive to pituitary gonadotropin and 10 times as sensitive to chorionic gonadotropin.

These facts naturally led to an investigation of the possibility of the use of the male Xenopus laevis for the diagnosis of pregnancy. However, the animal proved unsatisfactory for such tests. The difficulties encountered and possible explanation of them will be discussed in another paper.

Ample evidence existed from previous work suggesting the possible use of other species of male frogs or toads. In 1929 Houssay, Giusti and Lascano-Gonzales (1929) demonstrated that the female South American toad (Bufo marinus) reacted, in the form of ovulation, to the implanation of toad pituitary. In this same year Wolf (1929) accomplished the same result in the female Rana pipiens, also using homologous pituitary. The effectiveness of mammalian pituitary in the female Rana pipiens was not demonstrated until many years later

by Wright and Hisaw (1947). While these authors produced ovulation in the Rana pipiens, they called attention to the fact that other investigators, using essentially the same technic, had failed to achieve similar successful results. All this preceding work strongly suggested the probable universality of the reaction of frogs and toads to gonadotropic hormone of both amphibian and mammalian origin.

Based on this work a pregnancy test was recently described by Mainini using a male South American toad (Bufo arenarum Hensel) (1947). This toad, under the conditions of the investigation, appeared to have value in the routine diagnosis of pregnancy. Ninety-nine pregnancy tests were run in parallel with the Friedman test. In 94 instances the rabbit and toad agreed. In 5 tests "inconsistent" results were obtained, the toad being positive in 3 and the rabbit negative; in 2 others the rabbit was positive and the toad negative. Whether these "inconsistencies" could be construed to mean 3 false positive reactions and 2 false negative reactions was not clear. Seventy-seven control urines obtained from children, menopausal women and normal male and female adults, etc. were consistently negative. However, some doubt as to the practical usefulness of the test was raised by the author since, as he stated, " . . . it might happen that the reaction of the toad could be different during other seasons of the year, i.e. during the mating period or variations of tempature."

It is the purpose of this paper to make a preliminary report on the use and reliability of another species of male amphibian, the common North American frog (Rana pipiens) in the routine diagnosis of pregnancy.

The use of this frog has many significant advantages such as (1) abundant supply, it being indigenous to wide areas of the United States, (2) low cost, since the animals cost in the vicinity of 10 cents and 2–4 frogs suffice for individual tests, and (3) it reacts to untreated urine within 1–2 hours. Analyses on 112 urines from known pregnant women and women suspected of being pregnant form the basis of this report. In every instance where unequivocal clinical evidence of pregnancy, such as can be found in a 5–9 month gestation, was lacking, parallel tests were performed on rats and rabbits. An additional small series of urines from normal girls having regular menses was also tested.

<div align="center">TECHNIC</div>

The technic of the performance of this test consists essentially in the subcutaneous administration of 5 cc. of urine to each of a pair of male frogs. The first morning urine is used as in other biologic tests for pregnancy. After simple filtration through ordinary filter paper to remove crystalline sediment and debris, 5 cc. of urine is injected subcutaneously into the dorsal lymph sac. No adjustment of pH or control of the temperature has been found necessary and the animals take this volume of fluid with great ease. Cloacal smears of the animal are then performed, beginning one hour later,

following the technic described in a previous report (Robbins, Parker and Bianco, 1947). It consists essentially of aspirating the fluid obtained from the external cloacal orifice with a glass capillary pipette. It is unnecessary to enter the cloacal cavity since ample fluid is always obtainable at the outlet. The cloacal fluid thus obtained is examined, as has been described, in a hanging drop preparation under reduced light. The spermatozoa are very readily seen, more easily than those of the Xenopus laevis, since they are considerably larger and the reactions are usually all or none, producing either large numbers of sperm (Figures 1 and 2) or none. It is important to note that the pres-
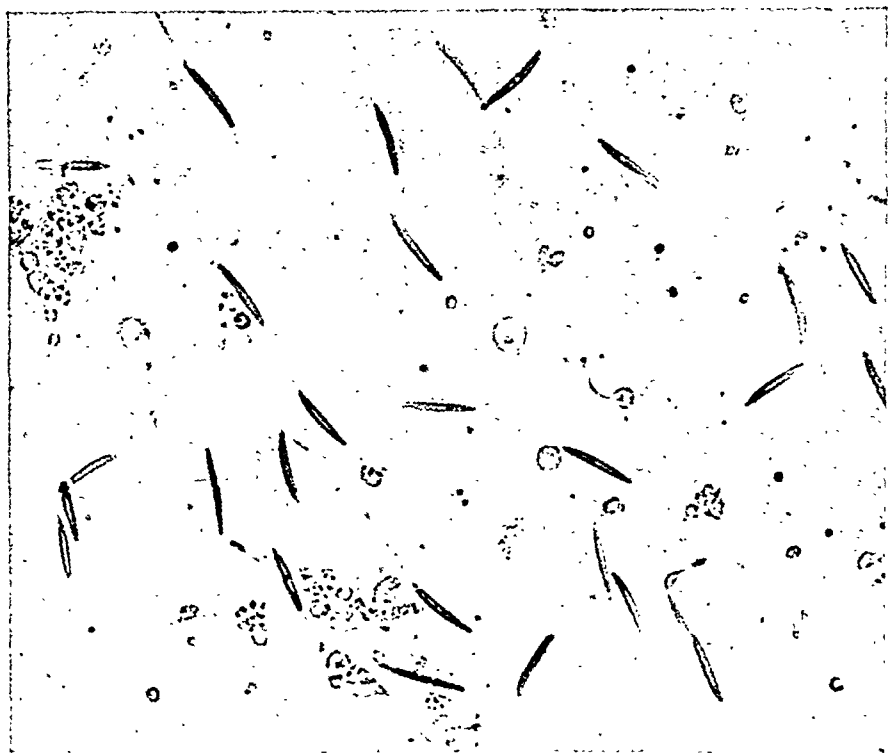
Fig. 1. The spermatozoa as seen in an unstained preparation under reduced light. ×800.

ence of any sperm, motile or not, is indicative of a positive reaction. When the first cloacal examination is negative it should be repeated in an hour. In the series of urines here reported positive reactions have never been delayed more than 2 hours. If a reaction does not develop within this 2-hour period after the administration of the urine, it is safe to conclude that the test is negative. The possibility of missing a positive reaction by the use of hourly examinations is quite remote. Once a reaction develops it persists at peak levels for 1–2 hours, and can be demonstrated at considerably lower levels for 4–5 hours.

Some of the urines tested were toxic, killing both frogs. Usually this toxicity is apparent within one-half hour after the administration of the urine, the frogs being either very sick or dead. In such cases the 5 cc. of urine is di-

urine. Methods of detoxification of urines are sorely needed for all bio-assay technics. Work on this problem is being continued.

As can be seen from the technic described, it has been customary to not re-use the frogs. This fact does not imply that re-use of the animals is impossible or not feasible, since on rare occasions individual frogs that have been used on negative tests have been successfully used over again with completely satisfactory results. However, since these amphibia are difficult to feed, living chiefly on live insects, and since under laboratory conditions their metabolic processes are stepped up over hibernating levels, it has been considered wisest to not permit inanition or wasting to develop by prolonged use of these animals. The one occasion when frogs were used that had been kept under usual laboratory conditions for several months without attempts at feeding proved beyond a doubt that such animals were completely unsatisfactory for use, since they failed to react to urines that gave positive results in frogs not subjected to long periods of laboratory storage. The fact that these amphibia inhabit almost the entire United States, save for a narrow zone along the Pacific coast, makes them readily available in this country.

Sufficient time has not elapsed to permit study of the problem of the use of these animals throughout the year. The Rana pipiens used in this investigation, carried on in the winter months, were obtained from Vermont where they had been kept in artificial hibernation at the bottom of a lake. Since hibernation is presumably the period of lowest metabolic activity and re-activity, it seems reasonable to assume that they should prove utilizable throughout the year. What precautions, if any, may have to be taken during their breeding season are as yet not known.

SUMMARY

A new test for pregnancy, utilizing the release of spermatozoa by the male Rana pipiens on stimulation with mammalian chorionic gonadotropin is described.

Its advantages are many. Small amounts (5 cc.) of whole urine can be administered directly to each frog, making the test very simple. Reactions to positive urines develop sometimes within a half-hour and invariably within 2 hours, producing a test considerably faster than any other previously described biologic test. The animals are widely available throughout the continental United States and as a result are extremely cheap; thus the cost of the test is most reasonable.

In a carefully controlled series of 122 urines no false positive reactions were encountered. One false negative reaction occurred on a urine early in the period of gestation.

The ease with which a diagnosis of pregnancy can be established with this test makes possible the adoption of this technic in any laboratory, however small. The rapidity that it affords makes of the test

an emergency procedure of possible benefit in difficult diagnostic problems.

ACKNOWLEDGMENTS

The authors wish to express their grateful appreciation to Miss Phyllis D. Bianco and Miss Gloria E. Mannix for their unstinting technical assistance.

ADDENDUM

Since this paper was submitted for publication P. B. Wiltberger and D. F. Miller (*Science*, Vol. 107, p. 198, 1948) described the use of Rana pipiens as a test for pregnancy and found it to be of high diagnostic value.

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EXPERIMENTAL DIABETES PRODUCED BY
THE ADMINISTRATION OF GLUCOSE

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A PERSISTENT INCREASE in the concentration of blood glucose has frequently been considered a factor in the production of damage to the islands of Langerhans. Some of the experimental data suggesting that this may be so is as follows: (a) The experiments of Allen (1922) and Homans (1914) indicate that in subtotally depancreatized dogs or cats a period of hyperglycemia precedes the occurrence of hydropic degeneration of the beta cells. (b) In dogs (Dohan, Fish and Lukens, 1941) or partially depancreatized cats (Lukens and Dohan, 1942) injected with crude anterior pituitary extracts hyperglycemia precedes the hydropic degeneration of the beta cells. (c) The hydropic degeneration produced by anterior pituitary extract may be prevented or reversed by means of diet, insulin or phlorizin (Haist, Campbell and Best, 1940; Lukens and Dohan, 1942; Lukens, Dohan and Wolcott, 1943). All these measures tend to maintain a normal concentration of blood glucose.

These and other considerations led us to attempt the production of hydropic degeneration and permanent diabetes by maintaining an increased blood glucose concentration by glucose injections. Previous experiments (Jacobs and Colwell, 1936; Astwood, Flynn and Krayer, 1942; Dohan and Stengel, unpublished) using the intravenous route suggested that intraperitoneal injections might be more satisfactory. Cats were found to tolerate such injections better than dogs, rabbits or rats. Part of this work has been reported in preliminary form (Dohan and Lukens, 1947). Previous efforts to influence the islands of Langerhans by the administration of glucose will be cited in discussing the results.

METHODS

Animals. Thirty-five apparently healthy cats of both sexes were used. Eight of these animals had approximately the splenic half of the pancreas removed under pentobarbital anesthesia. After several weeks, when preoperative weight, appetite and well being were completely restored they were considered suitable for experimental purposes. None of the operated cats developed spontaneous glycosuria.

Injections. The standard solution for injection contained 20% glucose and 0.9% sodium chloride. This was boiled for 5 to 10 minutes after preparation and kept in flasks in the ice box. After cooling, 100 mg. of thiamine hydrochloride was added to each liter of glucose solution. This solution was warmed to body temperature before injection and all injections were made intraperitoneally through the shaved and washed abdominal wall. Injections were usually made about 8:30 A.M., 4:30 P.M., and 10:30 P.M. and the dose varied from 50 cc. to as high as 420 cc. in 24 hours. Smaller and less frequent doses were used in the first few days. The clinical conditions of the animal, distention of the abdomen, glycosuria and glycemia were used as indications of the amount and frequency of injections.

Diets. In general the cats were fed measured diets of ground meat with a few grams of powdered yeast, cod liver oil and bone ash added. At times the supplements were omitted or canned salmon was offered as an inducement to eat. Usually the appetite was greatly reduced during the period of injection.

Chemical. The cats were kept in metabolic cages and urines were collected in the usual manner. Blood samples for glucose determination (Somogyi, 1945) were taken from the ear. The majority of such observations were made on samples taken just prior to the intraperitoneal injections and so represented the lowest values in the 24 hour period. For these values the term *pre-injection* blood glucose will be used. In some animals *pre-injection* blood glucose was determined before every injection, in others once or twice daily to ascertain the response and adjust the dose of glucose.

The following analytical methods were employed: urinary glucose (Benedict, 1911), cholesterol (Bloor, 1916), fatty acid (Stoddard and Drury, 1929), glycogen (Cori, 1932), absorbic acid (Roe and Kuether, 1943), galactose (Raymond and Blanco, 1928).

Histological. At autopsy the tissues removed for histological examination were fixed in 10% formalin. The pituitary and an additional section of the pancreas were placed in Zenkers bichromate solution without acetic acid. The Zenker fixed tissues were washed at 8–12 hours and the pancreas was stained by the Bensley-Mallory method; the pituitary by Mallory's method Hematoxylin and eosin stains were used for the other tissues.

<div align="center">RESULTS</div>

The course of hyperglycemia and the production of hydropic degeneration of the islets during glucose administration

Thirty-five cats were given 1 to 3 intraperitoneal injections of 20% glucose in 0.9% sodium chloride solution per day. Of these, 10 developed hydropic degeneration of the islands of Langerhans, and of this group 3 exhibited persistent diabetes after the cessation of glucose administration. Twenty-five cats failed to develop hydropic degeneration of the islands of Langerhans. Many of the animals, as will be discussed later, developed a syndrome of anorexia, ataxia, weakness and death. The results will be illustrated by the course of events in a single cat.

Figure 1 illustrates the food intake, the glucose injected and excreted and the daily minimal (i.e., *pre-injection*) blood glucose concen-

trations in a normal female cat (G-13) injected for 27 days. It will be noted that the amount of glucose injected was gradually increased from 10 grams the first day to 72 grams per day for the last 4 days before autopsy. After the second day injections were given three times a day at approximately 8, 6 and 10 hour intervals. After the fourth day of injection the majority of values for blood glucose were above 200 mgm. per 100 ml. During the last half of the period there was an increased proportion of injected glucose excreted in the urine.
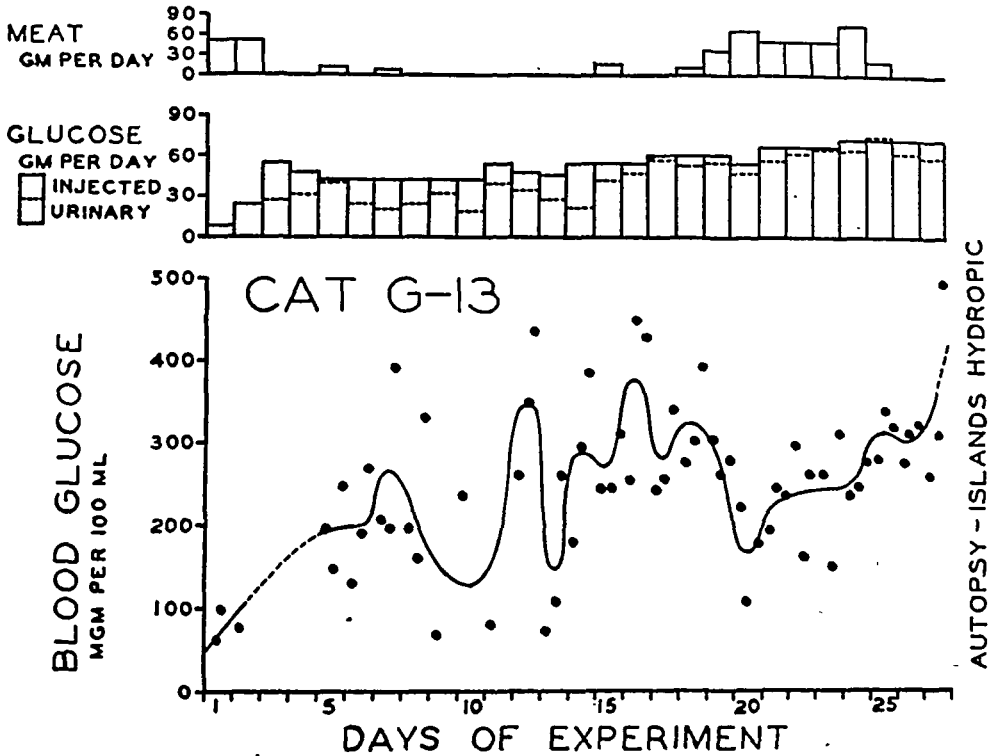
FIG. 1. The Production of Hyperglycemia and Islet Damage by the Administration of Glucose. This normal female cat was injected with 20% glucose in 0.9% saline solution for 27 days. Injections were given 3 times a day after the second day. Black dots indicate the blood glucose concentration just before each injection of glucose. The curved line indicates the averages of the daily observations. The hydropic degeneration of the islands of Langerhans is illustrated in Fig. 3. See also Tables 1 and 2.

For the 3 days prior to autopsy the cat excreted 89% of the total available glucose. In addition to the thiamine hydrochloride added to the injection fluid, yeast and cod liver oil were offered in the ground meat both before and during the period of glucose injections. As was true with the majority of cats, little food was eaten during the injection period. By the end of the 27th day the animal had lost 23% of its original body weight and was quite weak. Pentobarbital was given intraperitoneally and an autopsy was performed. The terminal blood glucose concentration (uncharted) was 1040 mgm./100 ml. 9 hours after the last injection of glucose. No evidence of peritonitis or other gross lesion was found. Sections from the pancreas showed marked
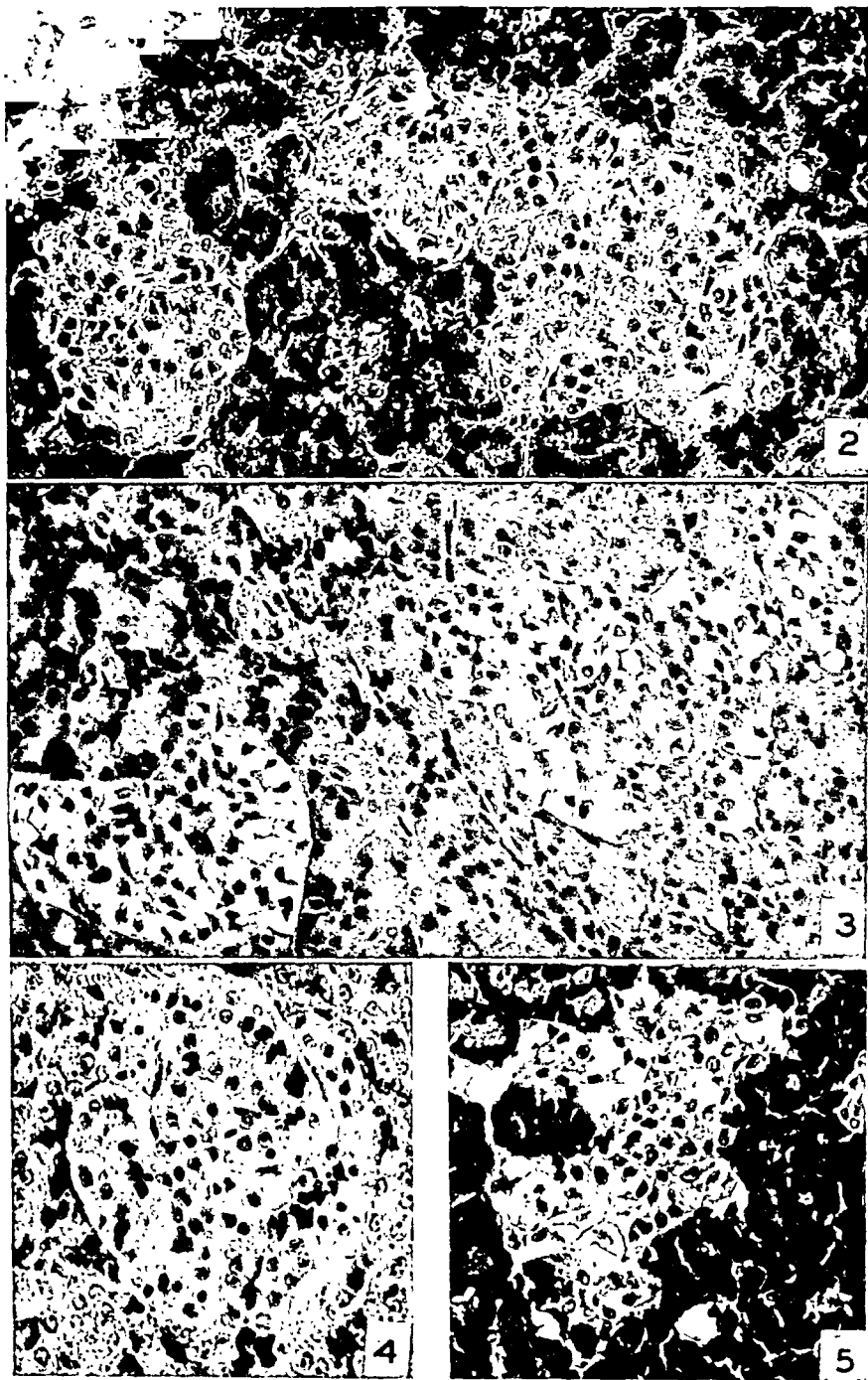
FIG. 2. Islands of Langerhans of normal cat. ×330.
FIG. 3. Islands of cat G-13 showing hydropic degeneration of beta cells. By Mallory stain the uninjured cells were alpha cells. ×330. See Fig. 1.
FIG. 4. Island of cat G-25. Slight degranulation of the cells when compared to the normal Fig. 2. ×330. See Fig. 7.
FIG. 5. Island of cat G-43. Irregular shape of island and small number of hydropic beta cells indicate beginning atrophy. ×300.

hydropic degeneration of the islands of Langerhans (Fig. 3). Sections of the liver, kidney, pituitary, thyroid, adrenal and ovary revealed no obvious abnormalities.

Relation of islet damage to the number of days of hyperglycemia

Table 1 shows the *days of hyperglycemia*, i.e., the number of days on which the morning blood glucose was 149 mgm. per 100 ml. or

TABLE 1. ISLET LESIONS RELATED TO DURATION OF HYPERGLYCEMIA

Cat No.	Days of hyper- glycemia[1]	Hydropic degeneration of beta cells	Weight of pancreas	Period of injection	Average[2] *pre-injection* blood-glucose
	days		gm.	days	mg./100 ml.
10 Cats Exhibiting Hydropic Degeneration of the Beta Cells					
5 Normal Cats					
G12	9	+	—	14	326
G13	19	++++	—	27	250
G19	20	++++	—	29	251
G16	33[3]	++++	5.0	38	216
G20	44	++++	10.7	104	198
Average	25		—	42	248
5 Partially Depancreatized Cats					
G 3	13	+++	2.05	30	209
G 8	14	++++	1.90	15	282
G46	29	+++	4.06	50	222
G43	31[3]	++++	5.50	52	270
G51	38[3]	++++	4.60	55	277
Average	25		3.62	40	252
25 Cats not Exhibiting Hydropic Degeneration of Beta Cells[4]					
Average	2	None	6.3[5]	9	115
Range	0–8	—	3.5–11.1	3–21	64–553

[1] Number of days on which the blood glucose concentration was greater than 149 mg./100 ml. immediately before the morning injection of glucose.
[2] Average of all blood sugars taken just before the intraperitoneal injections of glucose during the period of injection.
[3] Diabetes persisted after glucose administration was stopped.
[4] 22 Normal and 3 partially pancreatectomized cats.
[5] Pancreatic weight in 18 normal cats injected with glucose.

higher. These days were usually but not always consecutive. The period of hyperglycemia thus estimated is related to the status of the pancreatic islets. The morning blood glucose value was chosen as an index of the duration of hyperglycemia because the glucose concentration was determined more often at that time. Furthermore, in the great majority of instances it also proved to be the lowest of the 3 daily *pre-injection* values presumably because of the longer interval after an injection. It will be noted that the 10 cats which showed hydropic degeneration of the islets, had morning blood glucose values of 149 mgm./100 ml. or greater on 9 or more days, whereas the cats without lesions had "hyperglycemia" on only 8 days or less. The dura-

tion of hyperglycemia is also related to the total period of injection, to the average blood glucose of this experimental period and to the pancreatic weight. The effect of duration of the elevation of blood sugar is further illustrated in Figure 6, which relates the average of all *pre-injection* blood glucose values and the duration of injections to the islet pathology of the normal and partially depancreatized cats. The range of average blood glucose values for the cats with lesions was *198* to *326* mgm./100 ml. while for the cats without lesions
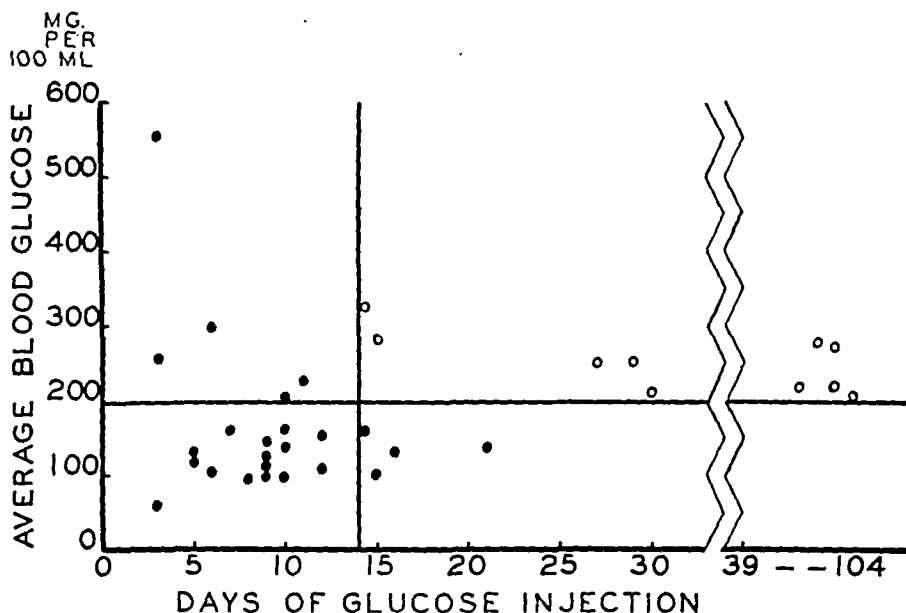
Fig. 6. Islet Lesions, *Pre-Injection* Blood Glucose Concentrations and Duration of Injections.
O = Cats with hydropic degeneration.
● = Cats without hydropic degeneration.

it was *64* to *553* mgm./100 ml. Particularly striking is the fact that 2 partially depancreatized and 3 normal cats exhibited average blood glucose levels of 196 mgm. or higher during the injection period of 11 days or less yet failed to show degeneration of the islets. For emphasis the chart has been divided into 4 quadrants. The cats without lesions but with high average *pre-injection* blood glucose concentrations are grouped in the left upper quadrant, and the 10 cats with lesions in the right upper quadrant. The symbols in the right lower quadrant show that those cats not exhibiting high average blood glucose concentrations even though injected for weeks, did not develop hydropic degeneration of the islands.

An example of a cat having high blood glucose concentrations for less than 9 days is presented in Figure 7. From the 3rd day to the 10th day inclusive this cat exhibited a moderate pre-injection hyperglycemia
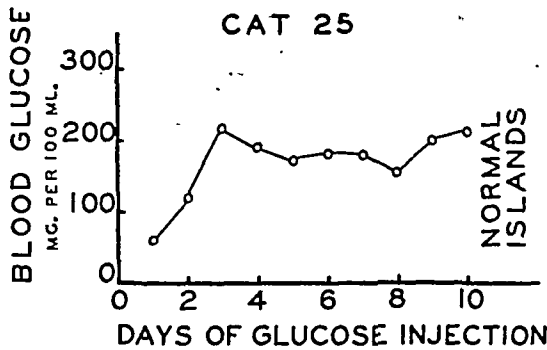
Fig. 7. Hyperglycemia of Short Duration without Hydropic Degeneration of the Islets. Normal female cat injected intraperitoneally 3 times daily with 20% glucose solution for 10 days. Initial weight 2.90 kg. Final weight 2.80 kg. Average daily dose of glucose was 14.3 gm. per kilogram of initial body weight. Circles indicate *pre-injection* blood glucose concentration. See Fig. 4.

yet failed to show hydropic degeneration of the islets as did those animals having hyperglycemia. A photomicrograph of the pancreas is shown in Figure 4.

Metabolism during the period of glucose injections

Some of the metabolic data collected during the period of glucose injections is tabulated in Table 2. As in Table 1, the cats with lesions

TABLE 2. METABOLISM DURING GLUCOSE INJECTIONS

Cat[1] No.	Period of injection	Average glucose injected	Average glucose Excreted	Available[2] glucose Excreted last 3 days	Average[2] caloric intake	Average[2] calories utilized	Weight Initial	Weight Final
	days	gm./k/d	gm./k/d	per cent	cal./k/d	cal./k/d	kg.	kg.
10 Cats Exhibiting Hydropic Degeneration of the Beta Cells								
5 Normal Cats								
G12	14	16.2	8.2	66	71	38	2.78	2.78
G13	27	18.9	14.6	89	80	32	2.78	2.15
G19	29	17.3	10.5	76	109	67	2.15	1.65
G16	38	11.4	10.0	87	100	60	3.38	2.75
G20	104	13.9	11.9	95	133	85	3.20	3.05
Average	42	15.5	11.0	83	99	56	2.86	2.48
5 Partially Depancreatized Cats								
G 3	30	8.5	6.6	107	34	8	3.98	2.65
G 8	15	5.8	4.8	95	23	4	4.82	3.50
G46	50	9.6	7.0	94	69	41	3.40	2.23
G43	52	9.4	9.0	89	96	60	3.15	2.75
G51	55	16.2	10.0	93	133	93	3.20	2.48
Average	40	9.9	7.5	96	71	41	3.71	2.72
25 Cats Not Exhibiting Hydropic Degeneration of Beta Cells								
Average	9	11.2	3.2	31	71	58	3.74	3.27
Range	3–21	6.2–22.7	0.4–8.8	0–75	25–129	13–124	2.1–4.8	2.2–4.8

[1] Tabulated in same order and grouped as in Table I.
[2] Gross approximation using standard tables and methods of calculation.

are listed according to the number of days upon which a morning hyperglycemia of greater than 149 mgm. % was present. The findings in the normal and partially depancreatized cats with hydropic degeneration are listed individually and may be compared with the average and the range of the findings in those cats without hydropic degeneration. *The average amount of glucose injected* varied considerably but, as expected, the amount necessary to keep the partially depancreatized cats hyperglycemic was less than that used in the normal cats. The wide range and lower average amount of glucose injected into the cats without lesions is a reflection of the fact that this group included most of those that became weak and ataxic as a result of the injections (see below) and, because of this, injections were often decreased or stopped.

As another indication of the severity of the diabetes the proportion of the available glucose excreted in the urine in the 3 days preceding autopsy is shown in the fifth column. It will be noted that the animals

TABLE 3. THE RESPONSE OF BLOOD SUGAR TO SINGLE GLUCOSE INJECTIONS

	Blood glucose		
	Pre-injection	4 hours	8 hours
	mg./100 ml.	mg./100 ml.	mg./100 ml.
Average	113 (40)*	347 (36)	236 (27)
Range	55–262	158–510	63–448
Average 5 highest	210	446	310
Average 5 lowest	58	286	137

* Figures in parentheses indicate number of observations.

with lesions excreted a high proportion of available glucose, ranging from 66% to 107%. The lowest amount excreted was found in cat G-12 which exhibited the least hydropic degeneration of the pancreatic islets (see Table 1).

A gross approximation of the *total caloric intake* has been made from the food ingested and the glucose injected. It will be noted that the caloric intake in general was the same or less than that usually ingested by healthy cats, i.e., the equivalent of 150–200 grams of meat per day for a cat weighing 3 kilograms. *Body weight* was lost in all but one of the cats with lesions and in most of the cats without lesions. This was due to glycosuria and decreased food intake. During the period of injections large amounts of intraperitoneal fluid were frequently present. At autopsy the animals were weighed before opening the abdomen so that the "final weight" was distorted by this gross disturbance of fluid balance.

The effect of intraperitoneal injections of glucose upon the concentration of blood glucose during the subsequent hours has been measured. In general, blood samples were obtained just before the injection and at the 4th and the 8th hour thereafter although a few complete hourly curves were made. The great majority of observations were made during the first to the 14th day of injections. Great

variability in effect was noted as was expected from the variation in the amount of glucose injected, the amount of fluid in the peritoneal cavity (resulting from the previous injections), the *pre-injection* blood glucose levels and general condition of the animals. The summary in Table 3 represents observations on 40 days in 20 cats. The 4th hour blood glucose value in particular indicates the transitory high values which the blood glucose concentration may attain.

The production of persistent diabetes

A persistent hyperglycemia and glycosuria has followed the period of intraperitoneal glucose injections in 1 normal cat and 2 cats with half of the pancreas removed. The course of events in the normal cat (G-16) is shown in Figure 8. This cat was injected 3 times a
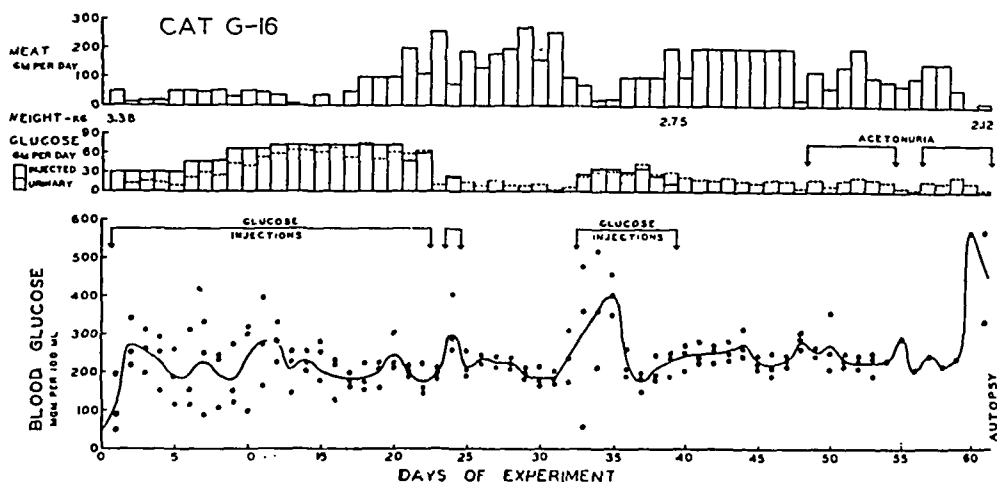
FIG. 8. Producton of Permanent Diabetes by Gluclose Injections. This normal male, cat was given 3 intraperitoneal glucose injections per day with 2 intermissions, over a period of 39 days. The black dots indicate the *pre-injection* blood glucose values during the period of injection and later the blood glucose at similar times when glucose was not being injected. The curved black line indicates the average of the daily observations.

day for a period of 39 days. A trial without injections was made from the 25th day to the 32nd day inclusive. Injections were then resumed on the 33rd day because of a blood sugar value of 58 mgm./100 ml. found that morning. The last injection of glucose was given on the 39th day. It will be noted that throughout most of the injection period a high concentration of glucose was present in blood samples taken just before each injection. These represent the lowest blood sugar values during the day. After stopping injections on the 39th day the cat was observed for 22 days during which it lost 0.67 kilograms of weight and the hyperglycemia and severe glycosuria continued. Slightly more than the calculated available carbohydrate of the meat diet was excreted during this period. On the 10th day after injections were stopped acetonuria was noted. By the 22nd day after the last injection (Fig. 8, day 61) the animal was obviously weak and de-

pressed and showed clinical evidence of acidosis. Pentobarbital was given intraperitoneally and autopsy was performed. The liver contained 1.04% glycogen and 21.2% of fatty acids, values similar to those obtained in untreated depancreatized cats. The blood obtained at time of autopsy also showed values to be expected in uncontrolled severe diabetes; thus the serum had a milky appearance and contained 1.60% of fatty acids, cholesterol was 243 mgm./100 ml., and urea nitrogen 105 mgm./100 ml. and the serum carbon dioxide content was 11 vols. per cent. Inspection revealed no evidence of infection and aside from the loss of subcutaneous fat and grossly fatty liver no gross lesions were noted. The liver weighed 95 grams, the pancreas 5.0 grams (normal) and the adrenals 710 mg. (above normal). Histological examination revealed severe hydropic degeneration of the islands of Langerhans. The liver was infiltrated with fat and other organs showed no noteworthy abnormality.

Two other cats, with half the pancreas removed, developed persistent post-injection diabetes. Data concerning these two cats, G-51 and G-43, may be found in Tables 1 and 2 which are supplemented by the following details. Cat G-51 weighed 2.48 kg. at the time glucose injections were terminated on the 55th day. After this it survived 8 days during which the average glycosuria was 77% of the available glucose of the meat diet. Anorexia and weight loss to 1.95 kg. preceded its death on the 9th post injection day and autopsy failed to reveal the cause of death. The liver fatty acids were normal (2.2%) and all tissues examined were microscopically normal except for the pancreas which showed marked hydropic degeneration of the islands of Langerhans. In the third cat (G-43) after 52 days of glucose injections severe diabetes persisted for the next 50 days. From the 50th to the 82nd day after glucose the animal continued to have glycosuria but in decreasing amounts. From the 83rd to the 97th day after glucose injection there was no glycosuria and normal blood sugar values were found. Despite the disappearance of diabetes there were marked changes in the pancreatic islands. They were decreased in number and the majority were very small with only a few hydropic beta cells remaining. Figure 5 illustrates one of the largest islands found. This animal which, during and after injections, had a total period of glycosuria of 6 months had atrophy of the islands resembling that seen late in pituitary-diabetes. The terminal disappearance of glycosuria is unexplained. (Although not found in this cat in the few sections examined, an adenoma of the type found in 3 other animals of this series might explain the results.)

The harmful effects of prolonged intraperitoneal administration of large amounts of glucose

A syndrome of anorexia, weakness of the hind legs and ataxia, progressing to severe weakness and death in animals injected intra-

venously, subcutaneously or intraperitoneally with large amounts of glucose for several days or more has been noted previously (Allen, 1913; Jacobs and Colwell, 1936; Astwood, Flynn and Krayer, 1942). These phenomena have appeared in all but a few of our animals without lesions. It was for this reason that larger amounts of glucose were not injected in these animals. It also occurred in a few of the cats showing hydropic degeneration of the islets. As anorexia was the rule during injection, early weakness was determined before each injection by the unsteadiness of the cat when dropped a few feet onto the
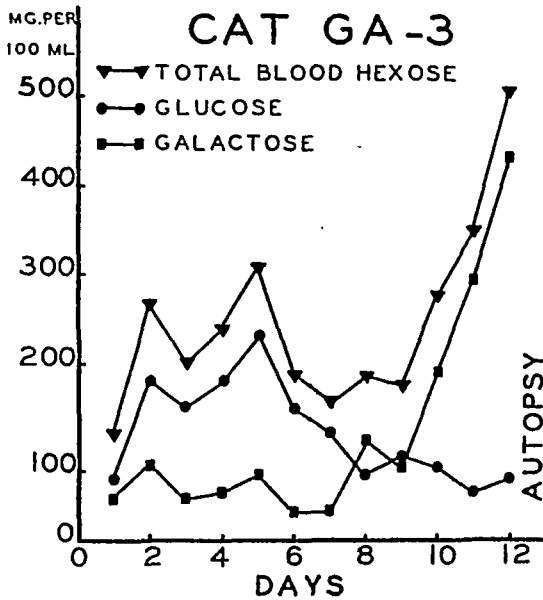
FIG. 9. This partially depancreatized cat without diabetes was injected with 20% galactose in 0.9% saline solution for 11 days. At autopsy on the 12th day the islets showed slight hydropic degeneration.

floor. However when weakness appeared it was usually progressive despite the cessation of injections and the use of the measures indicated below. Slight weakness of the hind legs might be followed in 12 to 24 hours by generalized weakness and death. Occasionally generalized convulsions appeared. Because of some similarity of the syndrome to thiamine deficiency large amounts of thiamine and cocarboxylase[1] were given at the first sign of weakness. These substances as well as riboflavin, niacin, pyridoxine, physiological saline, potassium chloride, sodium bicarbonate and adrenal cortical extract were given to various animals without conclusive evidence of beneficial effect. The pathogenesis of this syndrome remains obscure. We have also observed it during prolonged intraperitoneal injections of galactose. In this connection, Figure 9 suggests the failure to convert galactose to glucose in the 3 days before the syndrome developed. "Hepatic failure" of some kind may be the underlying cause of these

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[1] Kindly supplied by Dr. Randolph T. Major, Merck and Co., Rahway, N. J.

fatalities from glucose injection, although the liver which was regularly sectioned at autopsy showed no lesions. The central nervous system was not examined.

Galactose injections. To determine if hydropic degeneration of the islets of Langerhans is a specific response to the increased concentration of blood glucose a 20% solution of galactose in 0.9% sodium chloride solution was injected intraperitoneally in two cats. One normal cat was injected for 20 days with doses up to 72 grams per day but failed to develop *pre-injection* hyperhexosemia and at autopsy there was no hydropic degeneration of the islets. The other cat injected with galactose had had two thirds of its pancreas removed. 104 days before the first injection. During the period of injection frequent determinations of blood glucose and galactose concentrations were made. Figure 9 shows the results. During the first part of the experiment the concentrations of blood glucose were considerably greater than those of galactose but during the latter part of the 12 day period the reverse was true. The animal was sacrificed early on the 12th day as it had suddenly become weak and ataxic, exhibiting the same clinical picture shown by many of the cats which died during the injections of glucose. Autopsy showed the pancreatic remnant to weigh 2.16 gm. and the islets showed slight hydropic degeneration. It is clear that the conversion of galactose to glucose is so efficient that this method fails to solve the problem of the specificity of glucose as a cause of island lesions.

The response of the adrenal glands to the intraperitoneal injection of glucose

In response to a variety of stimuli there is an increased secretion of adrenotrophic hormone and hence of adrenal cortical hormone (Long, 1947). The measurement of this response by the fall in adrenal ascorbic acid and cholesterol, as described by Sayers, Sayers, Liang and Long (1946) and Sayers and Sayers (1947), has been employed. In Table 4 the data on rats are in fair agreement with the results obtained by Sayers and Sayers (1947) after other stimuli such as cold

TABLE 4. ADRENAL ASCORBIC ACID AND CHOLESTEROL AFTER INTRAPERITONEAL GLUCOSE

Conditions	No. of animals	Adrenal wt. mg./100 gm. body wt.	Ascorbic Acid mg./100 gm. fresh adrenal tissue	micrograms per 100 gm. body wt.	Cholesterol mg./100 gm. fresh adrenal tissue	mg./100 gm. body wt.
Normal rats	7	20.6±0.5*				
Rats; 35–60 mins. after glucose	4	16.6±0.3				
Normal cats	5	13.6±0.5	388±4.5	79.0±1.0		
Cats; 1½ hrs. after glucose	14	16.2±0.5	295±8.0	48.8±1.9		
Normal cats	7	16.4±0.8	102±2.1	14.1±0.5		
Cats; 1½ hrs. glucose	6	17.3±0.8	88±1.3	13.9±0.2	4.34±0.12	0.66±0.02
					2.96±0.24	0.51±0.04

* Mean and standard error.

and adrenotrophic hormone. The 40% fall in adrenal ascorbic acid is significant. In cats, the concentration of ascorbic acid in the adrenals fell significantly but when the adrenal ascorbic acid per 100 gm. body weight was examined the fall was not statistically significant, because of the low adrenal weight in the control series. The decrease in adrenal cholesterol was significant for both units of measurement. In addition to these measurements, a few lymphocyte counts have shown consistent lymphocytopenia after the intraperitoneal injection of glucose. This lymphocytopenia conforms to the response to injury described by Selye (1946), Dougherty and White (1944) and by Freeman and Elmadjian (1946), the last authors having observed

TABLE 5. ADRENAL WEIGHTS OF CATS UNDER VARIOUS CONDITIONS

Conditions	No. of cats	Average body weight	Average adrenal weight	Adrenal weight / Body weight
		kg.	gm.	gm. per kg.
Normal	15	3.43 ± .18	0.586 ± .037	0.174 ± .010
Glucose Injections 5 to 105 days	11	3.34 ± .30	0.620 ± .056	0.183 ± .011
Anterior Pituitary Extract 6 to 11 days	6	2.96 ± .40	0.775 ± .135	0.250 ± .028
Hypophysectomized 11 to 85 days	6	3.19 ± .18	0.252 ± .023	0.080 ± .004

this response during glucose tolerance tests in man. All criteria considered, it was apparent that cats varied considerably in their reactions to intraperitoneal glucose. In other words, the injection of glucose appeared to be a weaker and less consistent stimulus of the adrenals than the carefully regulated procedures of Sayers and Sayers (1947). Similar chemical studies have not been made after prolonged glucose injections but Table 5 shows the absence of any significant change in adrenal weight in these animals. This is contrasted with the marked increase in adrenal weight in cats treated with crude anterior pituitary extract. The atrophy of the adrenal after hypophysectomy is included in the table for completeness. From Table 4 it is recognized that there is an increased activity of the pituitary adrenal cortical system but as Table 5 shows, this appears to be much less in degree after glucose than after pituitary extract. In this connection it may be noted that exposure to cold, noxious chemicals, etc., which ought to cause the characteristic lowering of adrenal ascorbic acid, failed to increase the glycosuria of diabetic rats in the experiments by Ingle and Nezamis (1943).

The possible local action of intraperitoneal glucose on the pancreas

It is possible that a local action of 20% glucose solution in the peritoneal cavity might have been a factor in causing changes in the islands of Langerhans but the following facts suggest that this is

not the case. (a) The prolonged injection of glucose without 9 or more days of hyperglycemia did not result in island lesions. (b) Hydropic degeneration was present throughout the sections examined and was not confined to the surface of the pancreas as might conceivably occur from local action of the glucose. (c) There were no degenerative changes in the acinar tissue of the pancreas or in other retroperitoneal organs. (d) Only one of the 10 positive cats had evidence of peritonitis whereas 4 of the 25 negative animals had evidence of peritoneal irritation (hyperemia, cloudy fluid) at autopsy. (e) Finally, as Table 6 shows, pancreatic tissue glucose was of similar magnitude after intraperitoneal and intravenous administration of glucose. In these experiments, duplicate samples were taken at 90 to 150 minutes after intra-

TABLE 6. EFFECT OF GLUCOSE ADMINISTRATION ON BLOOD AND PANCREATIC TISSUE GLUCOSE

		Normal cats	After intraperitoneal glucose[a]	After intravenous glucose[b]
		mg. per 100 ml.	mg. per 100 ml.	mg. per 100 ml.
Blood	Avg.[c]	104 (3)	622 (4)	849 (4)
Sugar	Range	93–107	394–937	716–1017
Pancreatic	Avg.[c]	35 (3)	178 (4)	145 (4)
Tissue	Range	33–39	66–240	107–220
Glucose				

[a] 50 to 100 ml. of 20 per cent glucose in saline. Glucose concentration in peritoneal fluid prior to washing abdominal cavity with saline ranged from 2.8 to 4.0 per cent.
[b] 10 to 20 ml. of 50 per cent glucose in saline.
[c] Figures in parentheses indicate number of animals.

peritoneal administration and at 20 to 90 minutes after intravenous injection of glucose. The abdominal cavity was thoroughly rinsed with warm physiologic saline before obtaining the pancreatic tissue after intraperitoneal glucose. A piece of pancreas (about 0.5 gm.) was then dried with gauze, minced with scissors, placed in 10 ml. of 0.4 per cent tungstic acid. After weighing and filtering, glucose was determined. The increase in tissue glucose in both groups of animals resembles the results of Palmer (1917) in dogs. All the points discussed above lead to the conclusion that the transperitoneal action of glucose on the pancreas is not an important factor in the production of hydropic degeneration of the islets.

Autopsy findings. In the pancreas of animals with hydropic degeneration of the islets, the lesions resembled those described after partial pancreatectomy, pituitary-diabetes or early spontaneous diabetes in the cat (Lande, 1944). Degranulation of the beta cells was the earliest abnormality. As this is sometimes difficult to distinguish, only the unequivocal hydropic degeneration has been reported (Table 1). Only one animal in this series was followed long enough to permit the development of atrophy. There was no inflammatory reaction in the

islands, no changes occurred in the duct cells and hyaline degeneration was not encountered. In the partially depancreatized animals a variable amount of scarring was present as would be expected but there was no evidence that this affected the remaining islands. Three animals had adenomas of islet or duct tissue. the exact nature of the cells being uncertain. One of these was the animal (G-20) which did not become permanently diabetic after 105 days of injection in spite of extreme hydropic change in the islands. Four animals had purulent peritonitis and showed no island lesions. In the others there was no evidence of inflammation of the pancreas from intraperitoneal administration of glucose. All livers sectioned were normal except that of G-16 which was fatty. Kidneys, heart, ovaries, pituitary, thyroid and adrenals were normal by routine stains.

In 5 cats autopsied 1 to 4 days after the beginning of glucose injections and within 4 hours after the last injection, the liver glycogen ranged from 8 to 12%, values which are distinctly above normal. Lower values were found under other conditions. These levels of 8 to 12% glycogen in cats may be compared with 20 to 25% in dogs immediately after intravenous glucose (Butsch, 1934). Incidentally, Pavy (1894) noted that cats had only half as much liver glycogen as dogs after high carbohydrate feeding. Lundsgaard, Nielson and Orskov (1936) reported that perfused dog livers utilized some glucose but that no utilization was observed in cat livers. There is thus some evidence that glucose utilization by the liver may differ in the cat and dog. This is noted in connection with the failure of all workers to maintain prolonged hyperglycemia in the dog and the relative ability of cats to tolerate massive doses of glucose.

DISCUSSION

The role of species, level of blood glucose and duration of hyperglycemia in the production of damage to the islands of Langerhans will be better appreciated if certain previous studies are briefly reviewed. Although there is an extensive literature on the immediate effects of the administration of glucose by various routes, there are relatively few reports of attempts to give large quantities of glucose for long periods of time. In the early experiments, the absence of frequent blood sugar determinations leaves some uncertainty as to the physiological effect produced by the glucose administered. Later studies in which the blood sugar and the condition of the islets have been observed may be reviewed under two general headings.

A. The prolonged administration of glucose with slight hyperglycemia

Verzar and von Kuthy (1930) gave 50 to 150 grams of sugar daily to dogs by stomach tube for as long as 5 months. The fasting blood sugar was only slightly elevated (maximum 128 mgm. per 100 ml.) and glycosuria amounted from a trace to 14 grams per day. However,

glycosuria persisted for as long as a week after stopping glucose, and at the end of the experiments some of the glucose tolerance curves were mildly and temporarily diabetic. Except for the hyperglycemia (galactosemia?) in the course of the production of galactose cataract in rats (Mitchell, 1935) we are unaware that a sustained elevation of the blood sugar level can be produced by orally administered carbohydrate although glycosuria is readily produced. This was recently illustrated by Ingle (1946) who produced alimentary glycosuria in rats. The blood sugar was elevated during the day, hence the glycosuria but it was always normal before the morning feedings. The glycosuria disappeared after a few days in spite of continued increments in diet. The pancreas was not described. Woerner (1938) gave continuous intravenous glucose to guinea pigs for periods up to 28 days. With the exception of a single blood glucose of 270, the hyperglycemia did not exceed 147 mg. per 100 ml. In his later report (Woerner, 1939) he gave larger quantities of glucose. The longest survival was 8 days during which hyperglycemia was 200 mg. per 100 ml. or higher. He notes that "animals receiving 3 plus grams per kilo per hour continuously did not often survive much longer than 2 days." He recognized that "the functional state of the island cells may not always be accurately determined by the granule content of the cells alone," but he nevertheless concluded that when the blood sugar was high the majority of the beta cells showed beginning degenerative changes. The diabetic rat and rabbit have little or no hydropic change in the islands and diabetes in the guinea pig has apparently not been studied.

B. Marked hyperglycemia of short duration

The immediate effect of large amounts of sugar in the blood was reviewed and studied by Evans (1933) who found that single intravenous injections of glucose with marked hyperglycemia were harmless. Butsch (1934) maintained the blood sugar of dogs at very high levels for as long as 51 hours to measure the maximal capacity for glycogen storage. On histological examination the tissues were normal except for the large glycogen deposits in liver and muscle. Rabbits given 300 ml. of 10 per cent glucose per day subcutaneously died in 5 days (Bouckaert, 1934). Paralysis of the hind legs and its improvement when salt was given with glucose were observed. Jacobs and Colwell (1936) gave constant intravenous glucose for periods up to 6 days. However when hyperglycemia was maintained by large doses, the animals usually died within 48 hours. Death was attributed to acidosis, indicated by a marked fall in the CO_2 capacity before death. This was associated with gross hemorrhage in the pancreas and anterior pituitary but no specific lesions of the islands of Langerhans were seen. They described the symptoms preceding death under glucose administration as weakness and ocasional convulsions. Changes in granulation of the beta cells of guinea pigs (Gomori, 1939)

and mice (O'Leary, 1930) have also been seen after single doses of glucose. In dogs, Astwood, Flynn and Krayer (1942) also encountered early death with evidence of hepatic dysfunction revealed by bilirubinemia, impaired bromsulfalein excretion and hemorrhage. In a recent review, Houssay (1942) presents data on dogs given intravenous glucose for 4 days with definite elevation of the blood sugar. At autopsy on the 5th day there were no pancreatic lesions, a result which we would expect at this time interval. In summary, the use of species in which the response of the islands to glucose is uncertain, an inadequate level, or too brief a duration of hyperglycemia have been the chief obstacles in previous studies. In spite of this the careful observation of minor changes has provided a foundation for the experiments described herein.

The mechanism of production of hyperglycemia: comparison with anterior pituitary extract. In the initial development of hyperglycemia the use of glucose differs from that of pituitary extract in many respects. In the first 3 to 4 days, before lesions of the islands appear, crude anterior pituitary extract lowers the R.Q., inhibits the action of insulin on the blood sugar, promotes ketogenesis and thyroid and adrenal hyperplasia occur. There is probably a direct inhibition of the secretion of insulin (Anderson and Long, 1947; Houssay, 1942) and the insulin content of the pancreas is reduced (Best, Campbell and Haist, 1939). In contrast, the administration of glucose initially raises the R.Q., increases the efficiency of injected insulin, is antiketogenic and is not accompanied by thyroid or adrenal hyperplasia. Furthermore, glucose stimulates insulin secretion (Anderson and Long 1947) and carbohydrate diet maintains a high insulin content of the pancreas (Best, Haist and Ridout, 1939). Space does not permit the enumeration of other details. However, these facts suggest that in these two methods of producing hyperglycemia the physiological mechanisms preceding the hyperglycemia are quite different although the later development of hydropic degeneration and diabetes is essentially the same after both glucose and pituitary extract.

The mechanism of island injury. Allen (1913) concluded that the total caloric intake and body mass were important factors in the production of damage to the islets. In our animals with hydropic degeneration of the islands the caloric intake was equal to or less than that of normally fed cats and there was loss of body weight in all but one instance (Table 2). It thus seems that neither excess of calories nor increase in body mass were factors in the production of the lesions of the islands in the present experiments. This does not deny their importance under other conditions. The direct action of intraperitoneal glucose does not appear to be responsible. The diabetogenic property of certain pituitary and adrenal cortical preparations has been demonstrated in recent years and the pituitary-adrenotrophic system has been found to respond to glucose in these experiments. However

the lack of adrenal hypertrophy after glucose and the failure of other stimuli of adrenotrophic activity to aggravate diabetes (Ingle and Nezamis, 1943) lead to the tentative conclusion that this hormonal response is of little importance in the production of lesions. The degree of hyperglycemia and especially its duration have already been emphasized in relation to the development of island lesions. Whether or not there is some agent in addition to hyperglycemia, the events suggest that an excessive functional demand leads to the breakdown of function and to the anatomical changes of the islands. This cycle of functional stress followed by anatomical lesions has been repeatedly demonstrated in at least one other organ, viz. the kidney. The report of Medlar and Blatherwick (1937) is cited as an example of the production of renal insufficiency and renal lesions after unilateral nephrectomy. In the case of the islands, it is clear that hyperglycemia in the living animal is so complex a situation that other mechanisms must still be considered.

SUMMARY

Hydropic degeneration of the islands of Langerhans and persistent diabetes have been produced in cats by the administration of large amounts of glucose. The development of islet lesions was related to the degree and particularly to the duration of hyperglycemia. Neither excessive caloric intake, nor gain in weight, nor the local action of intraperitoneal glucose appear to be responsible. Although the adrenotrophic-adrenal cortical system reacts to intraperitoneal glucose, provisional appraisal of the problem suggests that this is less important than hyperglycemia in the production of islet injury. After hyperglycemia was established the course of the lesions and the development of diabetes closely resembled the sequence of events observed in experimental pituitary-diabetes. The contrast between the effects of glucose and of anterior pituitary extract in the first few days of their administration and the similarity of the events after hyperglycemia develops has been discussed.

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ORGAN WEIGHTS OF RATS RECEIVING HORMONE SUPPLEMENTS DURING RECOVERY FROM CHRONIC STARVATION

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REDUCED caloric intake has been shown to result in atrophic and functional disturbances of the hypophysis, the resulting reduction in pituitary activity being followed by secondary effects in other endocrine glands and organs. In a review of the literature, Stephens (1941) points out the histological and physiological evidence showing that chronic inanition is accompanied by structural and functional alterations of the pituitary. Both clinical and experimental data indicate that atrophic and degenerative changes in the thyroid, adrenal cortex, gonads, and sex accessories are associated with suppression of the anterior pituitary. Vollmer (1943) emphasizes that the atrophy of some of the endocrine organs in undernutrition is due less to the direct starvation of their tissues than to the failure of hypophyseal support. Trentin and Turner (1941) found that the mammary gland responded to estrogen at low feed levels, but that increased amounts of estrogen were required at such levels because of the physiological suppression of the pituitary during underfeeding. Mulinos and Pomerantz (1940a) summarize the picture by regarding the state of chronic inanition as one of "pseudohypophysectomy."

Although histological changes in the endocrine glands occurring in experimental undernutrition appear to be corrected by refeeding, as shown by Stephens (1940) and Jackson (1917), it does not necessarily follow that normal secretion and function are attained. Normal weight of some of the endocrine glands and other organs are recovered slowly or incompletely. In a study on prolonged refeeding of underfed young male rats, Jackson (1937) found that absolute weights were restored in only the brain, pituitary, kidneys, testes, and epididymides, while weight recovery in the skeleton, thymus, adrenals, heart, liver, and spleen were only relative.

Gonadotrophic effects of pituitary extracts and growth preparations on some of these organs in normal animals have been obtained by Riddle and Polhemus (1931), Friedgood (1936), Lee and Freeman (1940), LeRoy *et al.* (1942), and Hall and Selye (1945). Mulinos and Pomerantz (1941 a and b) employed pituitary implants in adult rats

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in a state of chronic inanition during continued underfeeding with the result that the thymus was decreased, the testes were increased, and the adrenal glands returned to normal weight. Smith (1930) made daily homeotransplants into hypophysectomized rats with the result that the decreased kidneys, adrenal cortex, thyroid, liver, spleen, and testes were restored or enlarged.

The foregoing constitutes the theoretical basis for the therapeutic use of pituitary extracts as an aid to recovery from inanition. Other endocrine related effects of inanition for which other hormone supplements might prove beneficial is brought out in several investigations. That there is a reduction in gonadotropin in chronic inanition and a consequent decrease in sex hormone secretion has already been shown. The atrophic histological changes in the testes and the atrophy of the accessory sex organs of undernourished rats as described by Mulinos and Pomerantz (1940b–1941c) suggested that injections of testosterone might make a substantial contribution to the recovery of these organs. Vollmer (1943) emphasizes that even though the rat continued to be underfed its genitalia are repaired by gonadotropin or testosterone. Moore and Samuels (1931) corrected the atrophic changes in the prostate and seminal vesicles of undernourished rats by the administration of testosterone. An increase in kidney weight in normal animals following injections of testosterone has been reported by Korenchevsky et al. (1933), MacKay (1940), and Luden et al. (1941).

Chronic starvation has been found by Jackson (1919) and Mulinos and Pomerantz (1941b) to result in a diminution in the weight of the adrenal glands. Although injections of adrenal cortex extract was not expected to influence the weight of the adrenal glands themselves, the pituitary-cortex and cortex-gonad relationship suggested that cortical supplements might be reflected in the weights of other glands.

The purpose of this investigation was (1) to check the work of Jackson (1915, 1925, 1937), Stewart (1916, 1918), and Stefko (1927) on the alterations in organ weights during chronic starvation and recovery, and to give analysis and interpretation to these changes, and (2) to determine the effects of supplements of adrenal cortex extract, testosterone, and growth hormone on organ weights, administered during realimentation and recovery.

METHODS

The studies made in this work were arranged in two separate experiments. In both experiments young male albino rats 30 days of age were employed in groups of ten, each group constituting an experimental unit. The rats were placed in individual cages and were underfed by restriction to a constant daily body weight ration of a qualitatively balanced diet. At the end of the selected periods of underfeeding the animals were put upon full and adequate rations for various periods of time. During recovery the supplements were adminsitered daily by intraperitoneal injection. At the close

of the selected refeeding periods the animals were anesthetized by nembutal, exsanguinated, and autopsied. In Experiment 1 each rat was underfed for 30 days and then refed for 35 days, receiving during the recovery a schedule of therapeutic injections as follows: Group I, 20 rat units—Growth Hormone ("Phykentrone," Squibb), Group II, 20 dog units—"Adrenal Cortex Extract" (Upjohn), Group III, ½ ml.—"Physiological Salt Solution" (Lilly). In Experiment II each rat was underfed for 90 days and then refed 60 days, receiving during the recovery a schedule of therapeutic injections as follows: Group IV, 5 rat units—"Growth Hormone." Group V, ½ mg.—testosterone "Oreton" (Schering). Group VI, ½ ml.—"Physiological Salt Solution" (Lilly).

In addition to these groups of refed rats, two groups of fully fed controls were observed during recovery; one was the same weight as the starved rats, and the other was the same age as the starved rats.

TABLE 1. AVERAGE ABSOLUTE WEIGHTS OF THE ORGANS OF RATS WHICH WERE CHRONI-
CALLY STARVED AND THEN REFED AND INJECTED WITH HORMONE SUPPLEMENTS
(Grams)

Experimental group	Pituitary	Adrenal	Brain	Spleen	Kidney	Testis	Epidydimides	Seminal vesicles	Thymus	Heart	Liver
Underfed 30 days	.0028	.0100	1.351	.212	.330	.390	.164	.160	.041	.217	2.40
Refed 35 days											
Saline	.0116	.0163	2.295	.705	1.032	1.237	1.010	.473	.417	.777	8.70
Cortex	.0116	.0156	2.260	.734	1.148	1.256	.994	.405	.419	.908	10.22
Growth	.0116	*.0246	2.219	*.844	*.966	1.103	*.726	* .334	*.627	.782	9.87
Normal control	.0139	.0205	2.403	1.355	1.164	1.354	1.200	.481	.370	1.025	11.65

(Percentage weight on body weight)

Underfed 30 days	.0048	.0172	2.620	.362	.564	.669	.273	.266	.070	.370	4.10
Refed 35 days											
Saline	.0049	.0067	.960	.296	.434	.520	.425	.199	.175	.327	3.66
Cortex	.0047	.0063	.940	.297	.465	.510	.405	.164	.169	.368	4.14
Growth	.0049	*.0103	.933	*.352	*.405	.460	*.303	*.140	*.262	.329	4.15
Normal control	.0047	.0070	.815	.460	.396	.460	.410	.164	.147	.350	3.96

* Indicates statistical significance.

In order to obtain data relevant to chronic starvation itself measurements covering all phases of this investigation were made on a group of rats sacrificed at the end of the starvation period.

After removing the blood as described above, the organs and glands were dissected out and their fresh weights determined on an analytical balance. The larger organs were weighed in regular weighing vials, but the adrenal glands, pituitary, and the thymus and seminal vesicles of the small normal rats and the underfed rats were weighed in vials of fixative the tare weight of which had been predetermined.

RESULTS

The numbers in the tables represent averages of a group of ten determinations on ten rats. The reliability of the averages was determined by plotting the range and mean deviation. Only differences that were found significant by this method are considered in the discussion.

The absolute and relative organ weights in chronic starvation, recovery, and recovery therapy are summarized in Tables 1 and 2.

DISCUSSION

The Effect of Chronic Starvation on Organ Weights. The effects of chronic inanition on the relative organ weights as determined by this experiment were for the most part the same as those found by Jackson (1915). There was no change in the average weight of the brain, an increase in the weight of the testes and pituitary, and a decrease in the weight of the thymus and spleen. The effects on the spleen and thymus as shown by these studies on rats are in accord with those observed on children by Stefko (1927). Disagreement appears regarding only the kidneys and liver, Jackson (1915) recording a slight increase in the weights of these organs, and this experiment showing no significant change in the kidneys and a decrease in the liver. While the

TABLE 2. AVERAGE ABSOLUTE WEIGHTS OF THE ORGANS OF RATS WHICH WERE CHRONI-
CALLY STARVED AND THEN REFED AND INJECTED WITH HORMONE SUPPLEMENTS
(Grams)

Experimental group	Kidney	Adrenal	Thymus	Brain	Testis	Seminal vesicle	Spleen	Liver	Pituitary	Heart
Underfed 90 days	.334	.0094	.026	1.41	.325	.015	.203	2.07	.0030	.20
Refed 60 days										
Growth	1.026	*.0225	.361	1.72	1.054	.472	.679	10.00	.0101	.83
Testosterone	*1.262	.0139	*.172	1.69	*.821	*1.122	.609	11.10	.0085	.95
Saline	1.308	.0165	.411	1.80	1.287	.548	.726	12.30	.0100	.93
Normal control (same weight)	.347	.0080	.179	1.34	.156	.032	.299	2.65	.0021	.28
Normal control (same age)	1.075	.0201	.309	1.80	1.304	.529	.962	12.60	.0119	1.06
(Percentage weight on body weight)										
Underfed 90 days	.605	.0170	.472	2.560	.590	.027	.363	3.76	.0054	.362
Growth	.431	*.0096	.1506	.724	.428	.192	.284	4.18	.0043	.349
Testosterone	*.532	.0059	*.0723	.713	*.350	*.581	.256	4.56	.0032	.381
Saline	.482	.0062	.1519	.673	.480	.201	.273	4.62	.0037	.343
Normal control (same weight)	.650	.0150	.3360	2.522	.291	.059	.562	4.95	.0039	.523
Normal control (same age)	.454	.0067	.1137	.605	.439	.177	.328	.422	.0041	.355

* Indicates statistical significance.

difference in results on kidney weights is likely due to the age of compared controls, that on the liver is more difficult to explain. The reduced liver weights of this experiment, however, agree with those found by Stefko (1927) in underfed children. The weight of the adrenal glands in the underfed rats was in nearly the same ratio to the body weight as that of normal rats of the same size, but is increased and consequently in accord with Stewart (1918) when compared with controls of the same age. Since the relative weight of the adrenals decreases with age in normal animals, this latter comparison is logical. The relative weight of the heart is also reduced with body growth so that when compared with controls of the same age there was no significant change as a result of starvation. On the other hand, the weight of the heart was relatively less as a result of underfeeding when compared with normal controls of the same size. Stefko (1927) observed a similar reduction in heart size in children suffering from chronic malnutrition. Consideration given to the age and size of the control rats does not otherwise alter the general effects of underfeeding as already

stated, except in the case of the brain. The brain of an animal starved in any way at any age always constitutes a greater per cent of the body weight than it does in a normal animal of the same age.

Since the weights of the kidney, brain, pituitary, adrenal glands, and testes in chronic starvation were in normal or greater than normal proportion to the body weight, it is concluded that these organs were "preserved" or "protected" during underfeeding in young rats. Probably the growth impulse of these organs overbalances the effects of caloric restriction.

The thymus, seminal vesicles, spleen, and heart, on the other hand, sacrifice their growth and contribute their substance in a proportion greater than other organs and body tissues. The reduction of the thymus is probably a case of vitamin involution, since Stoerk and Zucker (1944) found that in otherwise adequate diets, partial deficiency of vitamins of the B complex depressed the thymus weight. Sure (1938) also concludes that in B_1 deficiency there is atrophy of the thymus. Reduction of the seminal vescles in the chronic starvation of these rats is due to the diminished male sex hormone in connection with the inhibited testes. Mulinos and Pomerantz (1941c) have employed this interpretation to similar results in adult rats.

The relatively greater weight of the testes and pituitary at the end of the underfeeding period shows that these organs continued to grow slowly during starvation in spite of the suspended growth of the body and other organs. In view of the depressing effects of chronic inanition on the pituitary gland of adult rats, as shown by Mulinos and Pomerantz (1940a), and more specifically in view of the disturbed protein metabolism and reduced metabolic rates which occurred in the underfed rats of this investigation, one might have expected the pituitary to have had relatively less weight than the body. This is particularly true if consideration is given to the atrophic histological picture of the pituitary in chronic inanition in young rats described by Jackson (1917). However, an enlarged gland is not necessarily an active or over-active gland, as seen in the familiar response of the thyroid to endemic iodine deficiency; and the relative hypertrophy of the pituitary in the starved rats of this study may be just as indicative of a disturbed and inhibited function as if the gland had been reduced in size and weight.

The fact that the testes exhibited a marked weight gain in the presence of caloric restriction strongly suggests that the growth impulse in these organs was not independent of pituitary support. It may be, therefore, that the pituitary of young rats in chronic inanition continues to secrete a physiologically effective though reduced amount of gonadotropic hormone. A further evidence of some gonadotropic secretion may be found in the fact that in over 200 young male rats, chronically starved from 30 days' age, there were only two instances of an undescended testis.

Another possible instance of continued pituitary support is in the case of the adrenotropic hormone. While chronic inanition in adult rats is accompanied by an adrenal atrophy, as shown by Mulinos and Pomerantz (1941b) a marked adrenal hypertrophy occurred in the young rats of this investigation when compared with normal animals of the same age. It cannot be argued that the growth impulse prevents the adrenal atrophy in young rats, because the relative weight of the adrenal glands decreases with normal body growth.

The increases in the relative weights of the pituitary and adrenal glands during chronic underfeeding may be attributed to the lack of vitamin B_1 in the reduced daily ration. This explanation is proposed in view of the work of Sure (1938) who found that B_1 deficiency caused a marked hypertrophy of both of these organs.

The Effects of Refeeding on Organ Weights. The results of refeeding on the weights of organs corroborate those of Stewart (1916), with normal recovery of the brain, kidneys, and testes. There is also agreement on the relative recovery hypertrophy of the thymus and liver. The relative hypertrophy of the spleen in recovery as observed by Jackson (1925), however, is not confirmed by this experiment. Disagreement on this point is probably due to the tremendous individual variation in the weight of the spleen.

The recovery of the relative weights of the pituitary and adrenal glands as reported by Jackson (1937) in prolonged refeeding was observed in this experiment in rats underfed for 30 days, but was not observed in those underfed for 90 days. It appears therefore that the extent to which these two organs are restored depends upon the length of the undernutrition period.

While most of the effects of chronic inanition on young male rats are reversible, it is clear that these animals are not free from the stigma of the underfed condition. The effect on the weights of the kidneys, brain, testes, seminal vesicles, liver, and heart appear to be completely reversible, since these organs attain a normal absolute weight. The effects on the body weight, body growth, the weight of the adrenal glands, thymus, spleen, and pituitary are, on the other hand, only partially reversible since the recovery of these values was either lacking entirely or only relative to the partially recovered body weight.

The Effect of Growth Hormone on Organ Weights. An increase in the weight of the adrenal glands was affected by both small and large injections of growth hormone during recovery. This may have been due to traces of adrenotropic hormone which were probably present in the preparation employed. Friedgood (1936) and Hall and Selye (1945) obtained similar results with growth extracts on normal animals. The hypogenic effect on the adrenals obtained by Klieber and Cole (1939) with Evans' growth preparation may have been due to the very long period over which injections were made.

Large injections of growth hormone during recovery from the shorter starvation period produced enlargement of the spleen. This result is interpreted as indicating a "splenotropic" action on the part of the growth preparation used. Friedgood (1936) also noted an increase in the weight of the spleen in normal guinea pigs treated with an alkaline pituitary extract. However, using a similar extract, Riddle and Polhemus (1931) found no significant change in the spleen of young pigeons.

The growth hormone in large doses decreased the weight of the testes and sex accessories. This effect is interpreted as due to the inhibiting action on the pituitary gland of the growth hormone in general, and of the adrenocorticotropic hormone in particular. It is axiomatic that hormone supplements inhibit the gland which normally produced the hormone. Koneff (1944) has recently shown that the adrenocorticotropic hormone inhibits the anterior pituitary. Korenchevsky (1930) obtained a similar reduction in testes and sex accessories in normal animals with an alkaline pituitary extract.

A decrease in the weight of the kidneys resulted from the large injections of growth hormone. No measurements taken in this experiment and no published studies of a related nature made it possible to interpret this effect. In view of the results it can only be stated that the growth preparation used has an "anti-renotropic" action. This effect of the growth hormone on young rats recovering from inanition is in contrast with the kidney enlargement obtained with a crude pituitary extract on normal animals by Hall and Selye (1945).

No significant alteration in either the heart or liver appeared as a result of growth hormone administration. Other pituitary preparations have produced effects on normal animals which vary on either side of those obtained in this experiment. For example, extracts used by Lee and Freeman (1940) and by Riddle and Polhemus (1931) produced a liver hypertrophy, while those employed by Klieber and Cole (1939) and by LeRoy et al. (1942) resulted in liver reduction. Similarly, Hall and Selye (1945—report an enlargement of the heart with their extract, while Kliever and Cole (1939) found a reduction in heart size using Evans' extract. These disagreements undoubtedly arise from differences in the extracts, methods, and conditions employed.

Thus, while the growth hormone in heavy doses tended to increase and restore the normal weight of the spleen, it at the same time decreased the weights of the kidneys, testes, and sex accessories, and produced an abnormal hypertrophy of the adrenal glands. These results make it clear that large amounts of growth preparations are not indicated in recovery from chronic inanition. Since light injections of growth hormone, even after prolonged starvation, restored the normal weight of the adrenal glands without producing undesirable alterations in other organs, it may be that small amounts of prepara-

tions containing the adrenotropic hormone would be useful in effecting an earlier cortical recovery.

The Effect of Testosterone on Organ Weights. The testes were decreased in weight as a result of injections of testosterone during recovery. This effect was probably mediated through the inhibiting action of testosterone on the gonadotropic function of the pituitary gland. The interpretation has been proposed by Selye and Friedman (1941) and Shay *et al.* (1941) for a similar effect of testosterone on normal animals. There is also the possibility that the depressing action of testosterone injections on the testes may have been due to the oil vehicle rather than to the hormone itself. Cameron, Guthrie, and Carmichael (1946) found that daily injections of peanut oil for 17 or 18 days caused testicular atrophy and a decrease in rate of growth in the rat.

Testosterone administration produced a marked enlargement of the seminal vesicles; and since as stated above the testes themselves were inhibited by testosterone, it must be that the effect was due to the stimulating action of the hormone directly on the seminal vesicles.

A marked atrophy of the thymus occurred in rats treated with testosterone during refeeding. This effect was to be expected on the basis of the thymic involution which occurs normally in animals after puberty. Korenchevsky *et al.* (1933) obtained similar reduction of the thymus in normal animals injected with testosterone.

Rats receiving supplements of testosterone during recovery exhibited a significant increase in the weight of the kidneys. This action has also been observed on normal animals by Korenchevsky *et al.* (1933), MacKay (1940), and Luden *et al.* (1941). The mechanism and significance of this effect remains obscure.

The use of testosterone during recovery was attended with no measurable advantages, while presumably there were the undesirable effects of abnormal hypertrophy of the sex accessories, excessive thymic involution, and depression of the testes. It may be concluded therefore, that the administration of the male sex hormone in the amounts employed in this experiment is without benefit, if not actually contraindicated.

The Effects of Adrenal Cortex Extract on Organ Weights. Since the effects of the adrenal cortex extract on the thymus, seminal vesicles, and adrenal glands were very slight, the results are interpreted to be without significance. There may be in these small changes, however, a further evidence of the adrenal-gonad relationship.

The cortex extract produced no alterations in the weights of the organs which would justify either its use or disuse in recovery from inanition.

SUMMARY

Chronic starvation of both 30 and 90 days' duration decreased in young male rats the relative weights of the liver, thymus, heart,

seminal vesicles, and spleen; increased the relative weights of the adrenal glands, testes, and pituitary; and produced no change in the absolute weights of the brain and kidneys. The alterations produced were such that although refeeding resulted in the restoration of normal weights for the seminal vesicles and testes; the spleen, pituitary and adrenals failed to recover a weight in normal proportion to the body and the liver and thymus showed a relative hypertrophy.

The purpose of this investigation was to determine the effects of therapeutic supplements of adrenal cortex extract, growth hormone, and testosterone administered during recovery from chronic starvation upon the final organ weights of the rat.

One hundred rats, 30 days old and 50 grams weight, were brought to a state of chronic inanition by a qualitatively balanced constant body weight ration. They were then refed and groups of ten were injected with a different hormone. Two different periods of starvation and recovery were studied. Observations were made on body and organ weights on both starved and refed animals, as well as on the fully fed controls. All results were expressed as an average of ten animals.

The growth hormone administered during refeeding effected a normal absolute weight for the adrenals, but dosages adequate to increase also the weight of the spleen had a depressing effect on the testes, seminal vesicles ,and kidneys.

Injections of testosterone, while increasing the weights of the kidneys and seminal vesicles, decreased the weights of the thymus and testes.

The adrenal cortex extract produced no alteration in organ weights which could be regarded as significant.

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EFFECT OF VITAMIN DEFICIENCIES IN NEW HAMP-SHIRE CHICKS INJECTED WITH HIGH DOSES OF THYROXINE[1,2]

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HERTZ AND SEBRELL (1944) observed an interesting example of the interdependence of a dietary trace substance and a hormone-like factor; that is, between the metabolism of estrogens and the vitamin B complex, and further emphasized the importance of adequate nutrition for normal endocrine function. Hertz (1945) studied the direct quantitative relationship between the degree of oviduct response to stilbestrol and the dietary level of folic acid.

The vitamin requirements of hyperthyroid animals appear to be increased. In addition to the observations of Abelin *et al.* (1930), Abelin (1933), and von Euler and Klussman (1932) as quoted by Betheil *et al.* (1947), the following workers obtained evidence of increased requirements of vitamin A in the rat during experimental hyperthyroidism: Logaras and Drummond (1938), Sure and Buchanan (1937b). The same was found to be true for ascorbic acid requirements in the guinea pig by Svirbely (1935) and Sure and Theis (1939).

It may be assumed that an increased metabolic rate which results from the administration of a thyroid material may necessitate an increased dietary intake of members of the B complex. The results of many workers confirm such a hypothesis (Abelin, 1930) as quoted by Betheil *et al.* (1947), Cowgill and Palmieiri (1933), Himwitch *et al.* (1931, 1932). The partial protective effect against thyrotoxicity exerted by vitamin B_1 was first shown by Sure and Smith (1934), Sure and Buchanan (1937a) and confirmed by Drill and Sherwood (1938). Drill and Overman (1942) found that pyridoxine and Ca-pantothenate were required in larger quantities during experimentally induced thyrotoxicosis. Abelin (1945, 1946), as quoted by Betheil *et al.* (1947), feeding a crude ration, observed a beneficial effect when massive doses of Ca-pantothenate were administered to hyperthyroid rats. Ershoff and Hershberg (1945) were unable to alleviate the symptoms resulting from thyroid administration by greatly increasing the

Ca-pantothenate level of the ration. Ershoff and Hershberg (1945) found that rats fed a purified ration very high in the members of vitamin B complex known to be required by the rat grew poorly when desiccated thyroid was added. Betheil et al. (1947) have shown that very high levels of Ca-pantothenate as well as inositol exerted no beneficial effect on thyrotoxicosis.

Our object was to investigate further the relationships between vitamins and hormones with similar studies. The present experiment was planned to study the effects of various vitamin deficiencies on response to injected thyroxine.

EXPERIMENTAL PROCEDURE

Day-old New Hampshire chicks were reared in electrically heated batteries with raised screen floors. Feed and water were given *ad libitum*. The chicks were fed the respective diets for the full 4 weeks of the trials, body weights being recorded at weekly intervals. Table 1 gives the complete

TABLE 1. COMPOSITION OF BASAL RATION 110

Main ingredients	Per cent	Supplements	Mg./100 gm.
Glucose ("Cerelose")	61.4	Thiamine HCl	0.4
Casein (crude)	18.0	Riboflavin	0.8
Gelatin	10.0	Ca pantothenate	2.0
Soybean oil[1]	4.0	Nicotinic acid	5.0
Salts IM (Briggs, 1946)	6.0	Choline Cl	200.0
L cystine	0.3	Pyrixodine HCl	0.6
		Biotin	0.02
		Folic acid	0.2
		i-Inositol	100.0
		Para-aminobenzoic acid	0.2
		Alpha-tocopherol	0.5
		2-Methyl-1, 4-naphthoquinone	0.1
	Vitamin A in I.U.	1200[2]	
	Vitamin D₃ in A.O.A.C. units	170[2]	

[1] Substituted with Majola oil in the 3rd trial.
[2] Vitamins A and D₃ are fed by dropper weekly.

ration (110) used. The vitamin deficient rations were made either by completely omitting the vitamin concerned or by adding a low quantity as shown in Table 2. A low quantity of vitamin was added to the ration when necessary to prevent excessive mortality. At the end of 3 weeks, observations were made for the presence of naturally occurring black in the feathers. At that time a single injection of thyroxine was given.

The thyroxine solution was made such that one ml. of solution contained two mg. of crystalline thyroxine. The chicks were given one intramuscular injection of 0.5 ml. of thryoxine solution per 100 gms. of body weight on the 21st day. Thyroxine injections into New Hampshire chicks cause blackening at the base of the wing feathers (Juhn and Bernes, 1931; Juhn, 1933). This fact was the basis of the observations made on the response of thyroxine as affected by vitamin deficiencies. The incidence of mortality of chicks was recorded daily after injection on the 21st day. The records of blackening of the feathers were made on the 28th day. The blackening of the feathers was measured by the depth and width of melanin deposition in the feathers.

RESULTS

The results of all the experiments are summarized in Table 2. The typical deficiency symptoms of every vitamin were present in the respective group. The results obtained after several repetitions of the thyroxine treated and untreated chicks fed a ration deficient in different vitamins can be grouped as follows:

Feather-blackening.—Twenty-three out of 25 chicks of group 2 (complete ration—thyroxine injected) showed blackening at the base of the secondary and in some cases, primary feathers, which is the

TABLE 2. EFFECT OF VITAMIN DEFICIENCIES IN CHICKS INJECTED WITH THYROXINE

Group	Ration	No. chicks at 3 wks.	No. chicks at 4 wks.	Mortality	Average body weight 3 wks. gm.	Average body weight 4 wks. gm.	Gain, 3-4 wks.. gm.	No. of black feathers 3 wks.	No. of black feathers 4 wks.	Increase of black feathers at 4 wks.
1	110 (complete diet)	26	26	0	222	291	70	2	2	0
2	110+thyroxine (injected)	25	25	0	235	313	78	0	23	23
3	110 with low folic acid (15 gamma)*	15	12	3	113	101	-12	1	1	0
4	As group 3 +thyroxine	14	6	8	113	184	71	0	2	2
5	110 with low pantothenic acid (0.15 mg.)*	16	13	3	123	140	17	0	2	2
6	As group 5 +thyroxine	15	7	8	116	161	45	0	6	6
7	110 with low riboflavin (0.1 mg.)*	10	10	0	94	105	11	0	0	0
8	As group 7 +thyroxine	9	9	0	109	123	14	0	8	8
9	110 minus vitamin D	10	10	0	209	246	37	0	10	10
10	As group 9 +thyroxine	10	9	1	178	234	56	0	9	9
11	110 minus biotin	7	4	3	176	239	63	0	0	0
12	As group 11 +thyroxine	8	6	2	177	221	44	0	2	2
13	110 minus vitamin A	4	2	2	174	148	-26	0	0	0
14	As group 13 +thyroxine	4	0	4	170	—	—	0	0	0
15	110 minus vitamin E	8	8	0	210	257	47	2	6	4
16	As group 15 +thyroxine	8	8	0	206	251	45	0	8	8
17	110 with low B₁ (100γ)*	6	5	1	198	197	- 1	2	2	0
18	As group 17 +thyroxine	6	4	2	161	207	46	0	3	3
19	110 with low B₆ (100γ)*	7	7	0	145	188	43	0	0	0
20	As group 19 +thyroxine	8	6	2	133	172	39	0	6	6

* Amount per 100 grams of ration.

normal response of heavy thyroxine injection. Thyroxine injection of birds in lot 6 (low in pantothenic acid), lot 8 (low in riboflavin), lot 12 (deficient in biotin), lot 18 (low in B_1), and lot 20 (low in B_6) produced blackening of the feathers in a majority of birds. In groups 3 and 4 (low in folic acid), only 2 out of 6 birds had black feathers due to thyroxine injections. This low incidence may be partially accounted for by the slow rate of feathering caused by the deficiency of folic acid. Birds of both lots 9 and 10 (deficient in vitamin D) showed blackening of feathers, as has been reported by Glazener et al. (1946). However, the pigmentation of the feathers of birds from lot 10 (vitamin D deficient plus thyroxine) was of deeper intensity, distinct and in streak form whereas that of group 9 (vitamin D alone) was lighter and scattered. This experiment further indicates that a deficiency of vitamin E may produce blackening of feathers in itself. The thyroxine-treated group 16 (vitamin E deficient) showed blackening in the feathers similar to group 10 (vitamin D deficient plus thyroxine). In groups 13 and 14 (vitamin A deficient), incidence of feather blackening could not be recorded due to the death of all injected birds.

Mortality.—Birds of groups 3 and 4 were fed a ration low in folic acid. On thyroxine injection 8 out of 14 chicks died after the thyroxine injection. The pantothenic acid deficient chicks acted similarly, since 8 out of 15 thyroxine-treated birds died. Weaker birds died, leaving stronger ones in both the above groups, which probably accounts for the increase over the controls in average 4-week body weight noted below.

Body Weights.—It will be noted in Table 2 that in the first 5 series of comparisons, including the control lot, there is a consistent increase in gain of weight by the birds receiving the thyroxine injections. A total of 77 uninjected and 73 thyroxine-injected birds are involved in these comparisons. Although the differences between treated and untreated chicks by lots are not statistically significant, there is a consistent increase in favor of those lots receiving thyroxine.

In the remaining lots, the number of birds alive at the end of the experiment is too small to give any value to this measure.

Other Observations.—Another interesting effect of thyroxine injection was observed in chicks fed a ration low in pantothenic acid, in which a marked decrease in the deficiency symptoms of this vitamin (lot 6) was seen. Dermatitis observations were made during the second trial. Of the 6 surviving chicks of the uninjected group, all showed severe dermatitis. Of the 5 surviving injected chicks, only 2 showed mild dermatitis. Possibly the small amount of dermatitis which appeared might be eliminated entirely by the use of different levels of thyroxine, although there is no direct evidence for this as yet.

Further evidence for the effectiveness of thyroxine treatment was found in the fact that upon weighing the thyroid glands at autopsy those birds having received thyroxine consistently had smaller thyroid glands.

The average thyroid weight, calculated on the basis of mgm. per 100 gm. of body weight, was 8.38 mgm. for the controls and 4.65 mgm. for the treated group.

DISCUSSION

The data presented here show that thyroxine produces blackening of feathers in New Hampshire chicks even regardless of a deficiency of the following vitamins: pantothenic acid, riboflavin, thiamin, pyridoxine, and biotin.

The blackening of feathers produced in thyroxine-treated chicks fed a ration deficient in vitamins D and E was deeper than was produced in vitamin D- and E-deficient uninjected groups. This indicates that thyroxine metabolism was not affected in the absence of the above-mentioned B complex vitamins. In the absence of vitamins D and E, injected thyroxine activated the melanin cells to an even greater extent to deposit more black pigment in the feathers. In a majority of the thyroxine-treated chicks fed a ration low in folic acid there was no blackening. This may be accounted for in two ways—

either it was due to the slow rate of feathering or to the possibility that thyroxine metabolism was affected either directly or indirectly. As another possibility, perhaps the melanin-producing cells were affected directly by the folic acid deficiency.

In groups 4 and 6 (low in folic acid and pantothenic acid), higher mortality was observed as a result of thyroxine injection. For some reason the vitamin deficient chicks were susceptible to thyroxine toxicity. Therefore, in chicks these two vitamins appear to be needed in order to counteract the toxic effects of thyroxine.

The above results may be explained by the work of Abelin (1946) as quoted by Betheil et al. (1947), who showed a definite relationship between pantothenic acid and thyroxine in rats. He stated that thyroxine influences nearly all cells where pantothenic acid is present. This may possibly explain why injected thyroxine appeared to increase the body weights of some of the other groups (which had pantothenic acid in the ration).

The marked decrease in the deficiency symptoms of pantothenic acid in chicks, as a result of thyroxine injection, is another interesting vitamin and hormone relationship.

SUMMARY

In New Hampshire chicks fed a ration low in thiamin, pyridoxine, biotin, pantothenic acid, and riboflavin, injected thyroxine produces blackening at the base of the wing feathers. In vitamins D and E deficiency, thyroxine produced deeper blackening than was observed in D and E deficiency without thyroxine. Thyroxine caused high mortality in chicks fed a ration low in folic acid and pantothenic acid. Pantothenic acid deficiency symptoms in chicks were partially counteracted by thyroxine injection.

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THE ROLE OF BILE IN THE ABSORPTION OF STEROID HORMONES FROM THE GASTRO-INTESTINAL TRACT[1]

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SINCE BILE is required for the optimum absorption of cholesterol from the gastro-intestinal tract (Schonheimer, 1924) it has been assumed that bile is also essential for the enteral absorption of steroid hormones and related substances. On this assumption bile salts have been administered concurrently with steroid hormones to patients and experimental animals in an endeavor to enhance the potency of the latter substances when they are administered orally. Silberman, Radman and Abarbanel (1940) reported that testosterone propionate administered orally with bile salts was considerably more active in controlling the vasomotor symptoms of the menopause in women than was testosterone propionate alone. Hence these authors concluded that bile salts facilitate the absorption of testosterone propionate from the gastro-intestinal tract. Wells, Burrill and Greene (1940), on the other hand, were unable to show that the administration of desoxycholic acid increased the effectiveness of orally administered testosterone propionate in stimulating the prostate and seminal vesicles of the castrated rat. Selye (1943) also concluded that bile plays no important role in the enteral absorption of steroid hormones since the intragastric administration of desoxycorticosterone acetate, progesterone or testosterone to rats, in which the common bile duct had been ligated, produced a state of anaesthesia.

In the present experiments the role of bile in the absorption of steroids was assessed by determining whether the metabolites of dehydroisoandrosterone, progesterone and desoxycorticosterone appear in the urine, following the intragastric administration of these substances to rabbits in which bile was excluded from the gastro-intestinal tract by transection of the common bile duct.

METHODS

Each of the steroids studied was administered to six adult rabbits which were divided into groups of three. One group of unoperated animals served as a control and the other group consisted of rabbits in which the common bile duct had been cut between two ligatures four days prior to the administration of the steroid. In each instance the total dose of steroid was dissolved in 5 ml. of a 10% solution of ethyl alchol in propylene glycol and administered by stomach tube. Following the administration of the steroid the animals were placed in metabolism cages and the urine collected separately from each animal at twenty-four hour intervals.

Metabolism of dehydroisoandrosterone: Since preliminary studies revealed that the 17-ketosteroids excreted by the rabbit after the administration of

TABLE 1. EFFECT OF CONCENTRATION OF HYDROCHLORIC ACID AND DURATION
OF BOILING ON LIBERATION OF 17-KETOSTEROIDS FROM URINE OF
RABBITS TREATED WITH DEHYDROISOANDROSTERONE

Volume of HCl/100 ml. of urine	Duration of boiling	17-ketosteroids/100 ml. of urine
ml.	min.	mg.
2	15	2.05
2	30	2.52
5	15	3.65
5	30	4.5
5	60	5.2
10	15	5.15
10	30	5.05
10	60	5.8
15	15	5.8
15	30	5.5
15	60	5.3
20	15	5.7
20	30	5.5

dehydroisoandrosterone are in the conjugated form, it became necessary to establish the optimum conditions for the hydrolysis and extraction of these substances. To this end a total of 700 mg. of dehydroisoandrosterone were administered by gavage to three adult male rabbits whose urine was collected for four days. The urine was pooled and adjusted to pH7. Aliquots of 100 ml. of this urine were brought to the boiling point and different amounts (2 to 20 ml.) of concentrated hydrochloric acid were added and boiling continued for periods ranging from 15 to 60 minutes as shown in Table 1. Following rapid cooling the urine was extracted with ether; the neutral ether-soluble fraction was prepared in the usual manner and the kentonic steroids separated from the non-ketonic material by treatment with Girards' reagent T. Determination of the amounts of 17-ketosteroids present in the ketonic fraction was made be means of the Holtorff and Koch (1940) modification of the Zimmerman color reaction. As can be seen from Table 1 the maximum yield of 5.8 mg. of 17-ketosteroids was obtained either by the addition of 10 ml. of hydrochloric acid followed by boiling for 60 minutes, or by the addition of 15 ml. of hydrochloric acid with boiling for 15 minutes. The latter conditions were selected for the experiments herein described.

Each male rabbit received a single dose of 200 mg. of dehydroisoandrosterone. Urine was collected with hydrochloric acid as a preservative at twenty-four hour intervals for three days. The amount of 17-ketosteroids excreted in each twenty-four hour period was determined by the method described above.

Metabolism of progesterone and desoxycorticosterone acetate: The progesterone treated female animals each received 175 mg. of the steroid. Urine was collected under a layer of 75 ml. of butyl alchol containing 0.1 ml. of cresol, daily, for a period of 6 days. Each twenty-four hour collection of urine was extracted with butyl alchol; the butanol extracts were combined and the amount of sodium pregnanediol glucuronide present determined gravimetrically by the method of Venning (1938).

The female rabbits treated with desoxycorticosterone acetate each received 100 mg. of the steroid, The urine was collected and processed as described above for progesterone.

The identity of the precipitate isolated by the Venning procedure from the urine of the progesterone and desoxycorticosterone treated animals was established by melting point and by the isolation of pregnanediol-3 (α), 20, (α) from the products of acid hydrolysis.

RESULTS AND DISCUSSION

The rabbit, whose urine normally contains little or no 17-ketosteroids, excretes these substances following the administration of dehydroisoandrosterone. Previous studies (Hoffman and Desbarats, 1946) have shown that $\Delta^{3:5}$-androstadienone-17, dehydroisoandrosterone and etiocholanol-3 (α), one-17 are the 17-ketosteroids excreted by the rabbit treated with dehydroisoandrosterone. As shown in Table 2, the normal animals which received 200 mg. of dehydroisoandrosterone by gavage excreted from 38 to 44.3 mg. of 17-ketosteroids. The major portion appeared in the twenty-four period following the administration of the steroid. A smaller amount was excreted during the second day and by the third day the urine was practically free of 17-ketosteroids. Similarly the rabbits with transected bile ducts excreted 17-ketosteroids following the administration of dehydroisoandrosterone. The amount excreted varied from 34.1 to 54.9 mg. The completeness of the extra-hepatic biliary obstruction in these animals was established by the rapid development of jaundice and the occurrence of death within 8 to 35 days following the operation; at autopsy no accessory bile ducts could be demonstrated. Since these animals excreted 17-ketosteroids following the administration of dehydroisoandrosterone, it must be concluded that this substance was absorbed from the gastrointestinal tract in the absence of bile.

Four days were allowed to elsape after transection of the bile duct before administering the steroid in order to exclude the presence of bile from the upper gastro-intestinal tract at the time of treatment. To obviate the possibility that even after this period a small amount of bile might still be present, the experiment described above was repeated on animals in which the bile duct had been severed at least fifteen days before treatment. As before 17-ketosteroids appeared in

TABLE 2. EXCRETION OF 17-KETOSTEROIDS FOLLOWING ADMINISTRATION
OF DEHYDROISOANDROSTERONE BY GAVAGE TO NORMAL RABBITS
AND RABBITS WITH TRANSECTED BILE DUCTS

Rabbit no.	Condition of animal	Dehydroisoandros-terone administered	Amounts of 17-ketosteroids excreted			
			Day 1	Day 2	Day 3	Total
		mg.	mg.	mg.	mg.	mg.
89	Normal control	200	35.1	6.82	0.57	42.49
91	Normal control	200	33.1	3.89	1.01	38.0
92	Normal control	200	36.24	6.02	2.11	44.37
83	Transected bile duct	200	45.6	5.37	1.35	52.32
86	Transected bile duct	200	36.12	17.6	1.24	54.96
88	Transected bile duct	200	16.4	15.74	2.02	34.16

the urine following the administration of dehydroisoandrosterone.

The rabbit is known to convert exogenous progesterone and desoxycorticosterone acetate to pregnanediol which is excreted in the urine as the glucuronide (Heard, Bauld and Hoffman, 1941; Hoffman, Kazmin and Browne, 1943). In the present experiments, which are summarized in Table 3, the administration of 175 mg. of progesterone to normal female animals was followed by the excretion of 39.2 to 69.8 mg. of sodium pregnanediol glucuronide in the urine. Similarly the animals with transected bile ducts excreted 47.9 to 129.4 mg. of pregnanediol glucuronide after progesterone treatment. In these latter animals the identity of the precipitate was established by its physical properties and by the isolation of pregnanediol -3 (α), 20 (α) from the products of its acid hydrolysis. Here as in the case of the dehydroisoandrosterone experiments, the completeness of the interruption of the extra-hepatic biliary tract, was established by the clinical course and by the autopsy findings.

As shown in Table 4, similar results were obtained following treatment with desoxycorticosterone acetate. From 20.3 to 35.3 mg. of sodium pregnanediol glucuronide appeared in the urine of the normal animals given 100 mg. of desoxycorticosterone acetate by gavage. The animals with transected bile ducts treated the same way excreted 28 to 31.5 mg. of the pregnanediol conjugate which was identified in the manner described above.

TABLE 3. EXCRETION OF PREGNANEDIOL GLUCURONIDE FOLLOWING ADMINISTRATION
OF PROGESTERONE BY GAVAGE TO NORMAL RABBITS AND
RABBITS WITH TRANSECTED BILE DUCTS

Rabbit no.	Condition of animal	Progesterone administered	Pregnanediol glucuronide excreted
		mg.	mg.
79	Normal control	175	69.8
80	Normal control	175	39.2
81	Normal control	175	40.8
78	Transected bile duct	175	47.9
104	Transected bile duct	175	129.4
124	Transected bile duct	175	103.2

To exclude the possibility of the presence of residual bile in the gastro-intestinal tract of the treated animals, progesterone and desoxycorticosterone acetate were administered separately to rabbits in which bile duct transection had been performed at least fifteen days prior to treatment. Under these conditions pregnanediol was still excreted in the urine. Since pregnanediol appears in the urine following the intragastric administration of these two substances to animals in which bile is absent from the gastro-intestinal tract, it may be concluded that bile salts are not essential for their absorption.

TABLE 4. EXCRETION OF PREGNANEDIOL GLUCURONIDE FOLLOWING ADMINISTRATION OF DESOXYCORTICOSTERONE ACETATE BY GAVAGE TO NORMAL RABBITS AND RABBITS WITH TRANSECTED BILE DUCTS

Rabbit no.	Condition of animal	DCA administered	Pregnanediol glucuronide excreted
		mg.	mg.
118	Normal control	100	26.8
119	Normal control	100	35.3
121	Normal control	100	20.3
118	Transected bile duct	100	31.5
121	Transected bile duct	100	30.2
122	Transected bile duct	100	28.0

Although the amounts of metabolites excreted by the animals with transected bile ducts were as great or greater than the amounts excreted by the control animals, it cannot be concluded that the absorption of the steroids studied was as good in the experimental animals as in the control animals. At least two other factors may have played a role in determining the amounts of the administered substances which appear as metabolites in the urine. First, it has been observed that bile serves as a medium of excretion for the metabolites of steroid hormones. The administration of α-estradiol and testosterone to the human subject and the dog is followed by excretion in the bile of part of the metabolites of these substances (Cantarow, Rakoff, Paschkis, Hansen and Walkling, 1942, a.b.; Cantarow, Paschkis, Rakoff and Hansen, 1943; Paschkis, Cantarow, Rakoff, Hansen and Walkling, 1944). Therefore transection of the common bile duct, as was practiced in the present experiments, by interfering with a possible channel of excretion might have had some influence on the amounts of the metabolites excreted in the urine. Secondly, there is considerable evidence to indicate that the liver plays an important role in the metabolism of steroid hormones. In the rabbit, for example, it has been shown that the liver is capable of inactivating exogenous estrone (Biskind and Meyer, 1943) and progesterone (Masson and Hoffman, 1945; Engel, 1946). This is a possible factor in the present experiments since it is likely that the biliary obstruction which was produced would have caused liver damage, which in turn might have affected the metabolism of the administered steroids and thus indirectly have influenced the amounts of metabolites appearing in the urine.

Inspection of the results herein reported suggest that in the case of the progesterone-treated rabbits the amounts of pregnanediol in the urine of the animals with transected bile ducts were actually greater than in the urine of the control animals. This could be explained as a consequence of either or both of the two factors discussed above. Experiments are now in progress to determine the effect of liver damage, produced by prolonged extra-hepatic biliary obstruction, on the metabolism of steroid hormones and related substances.

SUMMARY

Crystalline dehydroisoandrosterone, progesterone and desoxycorticosterone acetate were administered by gavage to normal rabbits and rabbits in which bile was prevented from reaching the gastrointestinal tract by transection of the common bile duct. In each group of animals the amounts of metabolites of the administered steroids, excreted in the urine, were determined. It was found that the animals with transected bile ducts, treated with dehydroisoandrosterone, excreted as much 17-ketosteroids as did the normal animals similarly treated. The rabbits with biliary obstruction which were given progesterone and desoxycorticosterone acetate excreted pregnanediol glucuronide in amounts equal to or greater than control animals treated in the same way. It was concluded, therefore, that dehydroisoandrosterone, progesterone and desoxycorticosterone acetate can be absorbed from the gastro-intestinal tract of the rabbit in the absence of bile.

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LYMPHOCYTE DISCHARGE FROM THE ISOLATED RABBIT SPLEEN BY ADRENAL CORTICAL EXTRACT

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In an attempt to elucidate the mechanism whereby adrenal cortical hormones (ACH) increase the rate of lymphocyte dissolution within lymphoid organs (White and Dougherty, 1946), studies were undertaken to investigate the effect of ACH upon isolated lymphoid organs, perfused with whole blood. For the first studies, the effect of adrenal cortical extract (ACE) upon the isolated spleen was undertaken on the basis that if ACE acted directly upon lymphoid tissue to accelerate lymphocytic dissolution, this would be evidenced by a significant decrease in the number of circulating lymphocytes in the perfusion blood and by histological evidence of lymphocyte dissolution in the perfused spleen. When the first experiments were performed, contrary to expectations, it was observed that addition of ACE to the perfused spleen system, induced a prompt significant rise in the number of circulating lymphocytes, without affecting the number of circulating erythrocytes or polymorphonuclear leucocytes. This unexpected response of the isolated spleen to ACE was therefore investigated in detail.

The purpose of this paper is to present the results of these studies. The data indicate that the *in vitro* splenic response to ACE is specific in that ACE does not produce a similar effect upon either the perfused thymus, liver, or lung; and that perfusing the isolated spleen, with certain doses of estradiol dipropionate, desoxycorticosterone acetate, epinephrine, or sugar does not lead to splenic lymphocyte discharge.

METHODS

General: Rabbits of various strains, weighing between 3 to 4 kg. furnished the blood and organs used in the perfusion experiments. The isolated organs studied were spleen, thymus, liver and lung. All organs were perfused with whole heparinized blood which had previously been circulated through lung for at least 60 minutes to remove vasoconstrictor substances and possible emboli or foreign particles from the perfusion medium.[1] Spleen or thymus,

[1] It has been observed in the course of perfusing various rabbit organs (e.g. adrenals, ovary, testicle, kidney, etc.) with heparinized whole blood at a mean arterial pressure of 100 mm. Hg., which has not been passed through lung, that the organ perfusion is unsatisfactory. Thus, the initial blood flow through the organ is subnormal; as perfu-

unless otherwise stated, was perfused with a regulated pulsatile pressure (pulse rate 72 per minute) so that the mean arterial pressure near the tip of the cannula was maintained at a constant level (100 ± 10 mm. Hg.) Liver and lung were perfused at a non-pulsatile pressure equivalent to approximately 20 mm. Hg.

At varying time intervals, before and after the addition of the agent under investigation to the circulating perfusion medium, blood samples were withdrawn from the "arterial side" of the pump for analysis of RBC, WBC, differential white cell count and glucose (determined on Folin-Wu filtrates by a modification of the method of Schales and Schales 1945). Counting of RBC and WBC was performed, in duplicate, in the usual manner; differential analysis of leucocytes was made by counting 100 cells on air dried smears stained with Wrights' stain.

While the perfusion apparatus used in this study was designed to permit sterile perfusion of organs, it was not considered practical to attempt daily sterile perfusion experiments. To limit the influence of bacterial infection without utilizing tedious sterile perfusion technics (cf. Carrell and Lindbergh, 1938) penicillin and streptomycin were added to the perfusion medium. This combination of antibiotics effectively prevented the development of those types of bacterial growth in the perfusion system which otherwise contaminate it, and permitted short-term perfusion experiments virtually uncomplicated by the factor of bacterial infection.[2]

sion is continued the blood flow tends to decrease still further, and the organs become hemorrhagic, cyanotic, and edematous. These difficulties are largely avoided by passing blood through lung prior to using the blood for perfusion studies. The role of the lung in facilitating perfusion of isolated organs has usually been ascribed to removal of vasoconstrictor substances liberated in blood (cf. Hemingway, 1931), and to the retention of foreign particles or small emboli within the lung. However, it should be pointed out that the viscosity of blood is significantly reduced as the result of preliminary lung perfusion and it is possible that this lowering of blood viscosity is likewise important for the successful perfusion of isolated organs with whole blood. It may be mentioned that if serum is used as the perfusion medium instead of whole blood, it has been found that the preliminary lung perfusion is not necessary for successful perfusion of organs.

[2] Using a clean but not sterile technic for removal of blood and organs, and a nonsterile pump, for perfusion of lung, then spleen, it has been observed that without penicillin and streptomycin, after 5–6 hours of perfusion that 0.1 cc. of the perfusion medium placed on a nutritive agar plate and then streaked, gives rise to several hundred to a thousand colonies upon incubation at 37° C. for 48 hours. (Usually staph aureus, staph albus, B. Coli.) In six similar experiments wherein 2–5 Oxford units penicillin and 300–500 µg. streptomycin SO₄ per ml. blood was added to the pump, it was observed that 0.1 cc. blood removed after 5–6 hours of perfusion gave rise to 0, 0, 2, 4, 6, 6, and 10 colonies (mainly yeasts and less frequently fungi) upon agar plates incubated for 48 hours at 37° C. It may be mentioned in passing that these isolated colonies of yeasts and fungi did not grow upon the streaks, but only where the blood sample was dropped on the agar plate. The differences in perfusion experiments obtained with antibiotics are more striking with longer periods of perfusion. Thus, without antibiotics, blood became completely hemolyzed after 12 to 16 hours of perfusion and methemoglobin is formed. With penicillin and streptomycin it has been found in experiments with perfused adrenal glands that the majority of erythrocytes are still intact after 24 hours despite the presence of a yeast infection, and that the perfused organs showed only slight evidence of damage as revealed by histological examination. It may be mentioned in passing that if a factor effective against yeasts and fungi, but non-toxic to mammalian tissues, were available, combination of this agent with penicillin and streptomycin might permit sterile perfusion of organs without resorting to tedious aseptic and antiseptic technics.

Perfusion Apparatus: The perfusion apparatus illustrated in Fig. 1 was designed to permit the pulsatile perfusion of organs with whole blood for prolonged periods of time. Essentially it consists of two separate circuits, one for non-pulsatile perfusion of the lung to prepare the blood for subsequent perfusion; the other for pulsatile-perfusion of organs.[3] The apparatus consists of an organ chamber for pulsatile perfusion *A;* a blood reservoir *B;*
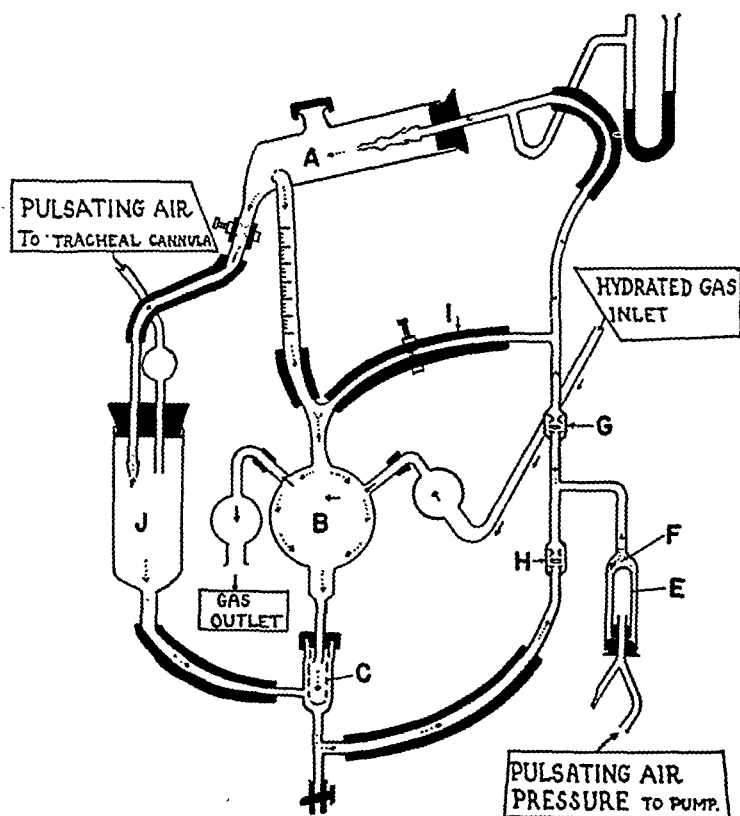
FIG. 1. A schematic drawing of the perfusion apparatus. The broken arrows show the course of blood circulation through the pump; the solid arrows show the course of oxygen through the apparatus.

a blood filter *C;* a pump, consisting of a rubber finger cot *E;* which is inserted into a pump reservoir *F* and is interposed between two one-way glass valves *G* and *H;* a pulse pressure regulator *I;* an organ chamber used for non-pulsatile perfusion *J;* and glass and other tubing which connects the separate parts of the apparatus. All glass in the apparatus is pyrex; the tubing used to connect the various parts of the apparatus may be either rubber

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[3] When serum, artificial media not containing cells, or highly diluted whole blood is used as the perfusion medium, preliminary perfusion of the blood through lung is not necessary. Accordingly, when these media are used for perfusion studies, the circuit for lung perfusion is not included; the apparatus is then more compact and sterilization is simpler. The shape or size of the organ chambers *A* and *J* may be modified depending upon the organ to be studied.

(Fenwal) with or without an inside coating of dimethyl silicone,[4] or Tygon flexible tubing. The apparatus is mounted in a constant temperature room maintained at 38° C., saturated with glycol vapor to sterilize the air.

The apparatus is actuated by means of compressed air (from a storage tank containing 20 lbs. per sq. in.) which is passed through a rotating valve (similar to that described by Lindbergh, 1935), thus creating a pulsating air pressure. The pulsating air is directed into the finger cot E of the pump, which then pulsates. As the finger cot expands, blood from the pump reservoir F is pumped past valve G into the line toward organ chamber A; on the off beat the finger cot contracts, sucking blood into F from the blood reservoir B, through filter C. Upon arriving at the organ chamber A, the blood can take either of two alternative routes depending upon whether a pulsatile or non-pulsatile perfusion is desired.

For non-pulsatile perfusions of lung and liver using pressures approximately 20 mm. Hg., the organ chamber A is used as an overflow vessel placed approximately 40 cm. above the cannula of the perfused liver or lungs in organ chamber J. This is achieved by having two outlets in organ chamber A—the lower outlet connecting with the cannula of the perfused lung or liver, while the other outlet takes the blood pumped in excess of liver or lung blood flow back to the blood reservoir B.

For a pulsatile perfusion the non-pulsatile perfusion circuit is clamped off; the organ to be perfused with pulsatile pressure is inserted in Chamber A, and the cannula is attached to the line. Blood pumped upwards from F then enters the nutrient artery of the organ, and then after passing through the organ runs down the floor of the chamber. The blood then flows down the sides of a calibrated tube connecting the organ chamber A and reservoir B, and thus passes into the blood reservoir B. Before the blood is recirculated it passes through a nylon stocking filter of small mesh (Fenwal).

Oxygenation of the blood is achieved by the following arrangement: The blood leaving organ chamber A returns to the blood reservoir as a film which adheres to the surface of the glass and other tubing. Upon entering the spherical blood reservoir, the blood spreads as a thinner film, and then flows down to mix with the blood in the reservoir B. By passing a continuous stream of 95% O_2—5% CO_2 gas mixture hydrated at the perfusion temperature, through the blood reservoir B, effective oxygenation of the moving film of blood is achieved without vaporation. Foaming of the blood during oxygenation is prevented by avoiding the passage of a gas mixture directly through the blood, and ensuring that the perfusion medium always flows on a surface. In this latter connection, it is important that the tubing connecting organ chamber A with the blood reservoir should have a minimal internal diameter of 10 mm.

The pump is interposed between two one-way valves G and H, and consists of a glass reservoir (length 10 cm. and 2.5 cm. I. D.), into which a finger cot is fitted by means of a rubber stopper. The maximal output of the pump is dependent upon the pulsation rate and the volume of the pump reservoir capable of being contracted by expansion of the rubber finger cot. The vol-

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[4] Rubber tubing can be coated with silicone polymers which then form a non-wettable surface which delays blood clotting (Jacques *et al.*, 1947). By coating all surfaces of a perfusion pump with silicone, it has been possible to perfuse liver with whole blood without the addition of any anticoagulants to prevent clotting in the system (Roche e Silva *et al.* 1947).

ume of the pump reservoir also determines to an important degree the minimal amount of blood necessary to fill the apparatus. The one-way valves (obtained from Macalaster Bicknell Company, Cambridge, Mass.) illustrated in Fig. 1 require special mention. When properly made, these valves do not stick, nor do blood cells settle out at the seat of the valve. They consist of a circular glass disc which sits upon a platform of glass at the end of the glass tubing; the surfaces of the disc as well as the platform are polished, not ground, thus minimizing the possibility of red cell rupture in opening and closing of the valve.

Mean arterial pressure measurement during organ perfusion was obtained with a damped Hg. manometer inserted into the line at a point just before the blood enters the organ chamber *A* and the nutrient artery of the organ (*cf.* Fig. 1). By using calibrated cannulae wherein the drop in pressure between this point in the line and the artery of an organ has been previously measured, the *mean* pressure in the artery of a perfused organ can be ascertained from the Hg. manometer.

Arterial pulse-pressure was not controlled during these perfusion experiments; however, visual examination of the artery of the organ during the course of perfusion afforded a gross qualitative index of pulse pressure. Where control of pulse-pressure is desired, the arrangement illustrated in Fig. 1, where a portion of the pump output may be short-circuited, provides a method of obtaining varying pulse pressure at a particular mean pressure. The amount of blood which is short-circuited is regulated by means of a Hoffman clamp.

Procedure for perfusion: A total of 110 to 150 cc. of blood, collected by heart puncture from two heparinized rabbits, was added to a flask containing 20 mg. heparin (Upjohn), 5000 Oxford u. penicillin sodium and 50 mg. streptomycin sulfate in a total volume of 3 cc. The contents of the flask were mixed and added to the perfusion pump. Immediately thereafter, the lungs and trachae of one of the exsanguinated rabbits were removed and cannulae were introduced into the pulmonary artery and trachea. The lungs were first flushed with Tyrode solution, introduced into the non-pulsatile organ chamber *J*, and were then perfused with the previously collected blood, for at least 60 minutes. During this period the lungs were inflated and deflated by a pulsating air current directed into the tracheal cannula. The average blood flow through rabbit lungs perfused under these conditions varied between 60 and 150 cc. per minute.

While the lung perfusion was proceeding, a third rabbit was prepared to furnish the organ for perfusion. The animal was anesthetized with pentobarbital and then injected intravenously with 10 mg. of heparin. The necessary blood vessel ligations were made; then the organ was removed, cannulated and flushed with Tyrode solution. After the lungs were removed from the apparatus, the organ to be perfused was inserted into the appropriate chamber. Thus, another lung or liver was placed in the non-pulsatile chamber *J* replacing the previously perfused lung; thymus or spleen was placed in the pulsatile organ Chamber *A*, and the non-pulsatile perfusion circuit was cut off by a clamp.

Spleen was prepared for perfusion by inserting a cannula into the abdominal aorta above the coeliac artery, ligating the aorta below the coeliac artery. In all splenic preparations the gastrohepatic artery was tied off; in some prep-

arations, all of the gastric and pancreatic branches of the lieno-gastric artery and vein were ligated; in others, the pancreatic and some of the gastric branches of the splenic artery were left open as bleeders. With both types of splenic preparations, it was noted that in approximately 25 per cent of the preparations, the circulation through the spleen rapidly became blocked, as evidenced by the cyanotic appearance of the organ and (where this was possible) by measurements of blood flow. These ineffectively circulated splenic preparations were discarded and are not included in the results to be reported here. In most splenic preparations, a variable amount of hemorrhage into the connective tissue surrounding the blood vessel occurs; however, this did not appear to significantly affect the circulation through the organ, as evidenced by coloration of the perfused spleen.

Thymus was prepared for perfusion by inserting a cannula into the descending portion of the thoracic aorta directed toward the aortic arch, ligating the ascending aorta, the subclavian arteries behind the internal mammary arteries, and the common carotid arteries above the superior thyroid arteries.

Lungs and liver were perfused in the ordinary fashion: liver via the portal vein, lung via the pulmonary artery.

In all instances, care was taken to ensure that no air emboli were present either in the cannula or the blood vessels of the excised organ. Furthermore it should be mentioned that in all of the perfused preparations the venous out-flow was not cannulated, but allowed to issue from the opened veins. Upon conclusion of the experiment, the perfused tissue was fixed in 10 per cent formalin for subsequent histological examination.

Sampling and addition of agents: "Arterial blood" samples were obtained with a hypodermic syringe by inserting the needle into a rubber connection at a point in the line just before the blood entered the nutrient artery of the organ. "Venous samples," when taken, were obtained from the outflow issuing from the organ by clamping the rubber tubing connecting the organ chamber *A* with the blood reservoir *B* and using a syringe and needle for sampling. Blood flow through the organ was measured (in those cases where all arterial bleeders were ligated) by clamping the same tubing, and measuring the time (in 0.1 second) necessary for the calibrated tube to fill to a particular volume.

Addition of various agents (sugar, hormones, etc) was made by injecting solutions through the rubber cap in the organ chamber *A: thus in these experiments all injected materials entered the venous side of pump*, and were filtered before entering the perfused organ. Water soluble agents added in this manner readily mix with the circulating blood. When agents dissolved in oil vehicles are added, the following course of events occurs: The blood issuing from the organ washes the oil solution into the blood reservoir *B* where, for the most part, the oil remains on the surface of the blood in the reservoir. Most of those oil globules which pass down the reservoir with the circulating blood, are trapped by the nylon filter and are prevented from entering the circulation.

Agents investigated: Two ACE preparations were studied, a lipo-adrenal cortex extract (Upjohn) and an aqueous extract (prepared by Endo Products Co.). The lipo-adrenal cortex extract henceforth referred to as ACE (oil) is dissolved in a cottonseed oil vehicle containing 1 mg. per cc. chlorobutanol

as preservative. The aqueous extract (1 cc. equivalent to 60 gms. fresh tissue) henceforth referred to as ACE (aq.) is dissolved in 0.9 per cent NaCl and contains 1:10,000 phenyl mercuric salicylate. In addition to these ACE preparations, desoxycorticosterone acetate and estradiol dipropionate (both .in a peanut oil vehicle) epinephrine hydrochloride (Parke-Davis) and glucose were tested to determine their influence upon splenic discharge of lymphocytes. ·

RESULTS

Before starting to examine the effect of ACE upon the perfused spleen, it was necessary to obtain information on three preliminary points concerning the formed elements of blood: (a) the effect of circulating the blood, with and without added ACE, through the apparatus in the absence of any organ; (b) the effect of preliminary perfusion of blood through the lung; and (c) the effect of spleen perfusion in the absence of added ACE.
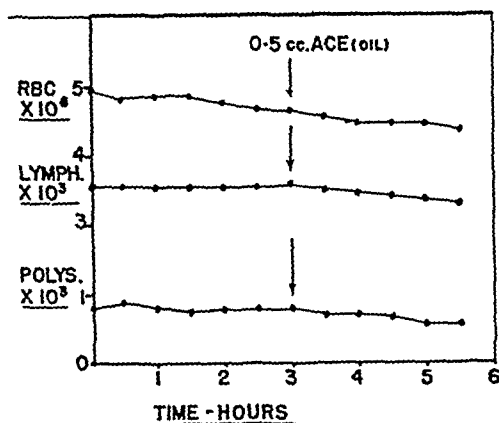
FIG. 2. The effect of circulating blood through the apparatus, upon the formed elements of blood, in the absence of an organ, before and after the addition of ACE. All values in terms of cells per mm.³.

The Effect of Circulating Blood through the Apparatus, with and without ACE. To determine the effect of the in vitro conditions of circulation upon the cellular elements of blood, whole blood was circulated through the apparatus in the absence of any organ in four experiments. In three of these experiments ACE (oil) was added to the apparatus after the blood had been circulated for several hours to test the possibility that ACE might directly affect the number of circulating red or white blood cells. Fig. 2 illustrates the results of a typical experiment of this type.

In this experiment the volume of the perfusion medium was 155 cc.; blood was pumped through a cannula at a rate of 180 cc. per minute; 24 mg. glucose was added every 30 minutes to maintain the blood sugar at a level of approximately 130 mg. per cent. The blood was, circulated for three hours without ACE, and then for an additional 2.5

hours after the addition of 0.5 cc ACE (oil). It will be seen that the number of circulating erythrocytes, lymphocytes and polynuclear leucocytes in the blood circulated through the system without an organ remain essentially unchanged, during the duration of the experiment.

In the other experiments, similar results were obtained as regards the minimal destruction or removal of erythrocytes and leucocytes in the course of recirculating blood through the apparatus *per se* with or without added ACE (oil) when the rate of flow through the apparatus was varied from 20 to 200 cc. per minute, or when the blood sugar level was permitted to fall without replenishment with sugar.

These results demonstrate that circulation of blood through the perfusion apparatus *per se* produces only minimal changes in the

TABLE 1. EFFECT OF PERFUSING BLOOD THROUGH LUNG UPON CELLULAR
ELEMENTS AND SUGAR CONTENT OF THE BLOOD

Exp. No.	Blood vol.	Av. blood flow	Initial				% Decrease as result of lung perfusion							
							30 min.				60 min.			
			RBC*	L†	P‡	G§	RBC	L	P	G	RBC	L	P	G
1	cc. 120	cc./min. 110	5.60	9.88	5.25	98	11	49	79	16	16	53	90	30
2	120	60	5.40	5.57	2.30	101	15	21	48	14	17	22	63	32
3	138	—	5.03	4.10	1.20	105	3	15	52	15	8	36	75	29
4	110	90	4.83	4.75	2.10	93	5	47	78	12	30	53	96	24
5	123	60	4.97	2.98	1.28	99	2	18	48	18	5	65	92	32
6	125	85	4.36	3.52	1.20	—	0	8	76	—	3	20	85	—
Mean							6	26	62	15	13	41	83	29

* RBC ($\times 10^6$) per mm.³.
† L is the absolute lymphocyte count ($\times 10^3$) per mm.³.
‡ P is the absolute polymorphonuclear leucocyte count ($\times 10^3$) per mm.³.
§ G is the blood glucose in mg. %.

number of circulating erythrocytes and lymphocytes, and that under these conditions ACE does not influence the number of circulating blood cells.

The Effect of Preliminary Lung Perfusion upon the Cellular Elements of Blood. Table 1 illustrates the changes in the formed elements of blood produced as a result of preliminary lung perfusion. It will be seen that as a result of passage of blood through the lungs for 60 minutes that the RBC count is reduced an average of 13 per cent, lymphocytes 41 per cent, and polymorphnuclear leucocytes 83 per cent; thus the leucocytes present in the blood after perfusion of lung are mainly lymphocytes.

Visual inspection of the lung reveals that certain areas become hemorrhagic and edematous during the course of perfusion; histological examination of these areas reveals endothelial ruptures with passage of mainly erythrocytes and a few leucocytes into the alveolar air spaces. It would therefore appear that the disappearance of a portion of the red and white blood cells during lung perfusion could be accounted for on the basic of this mechanism.

Effect of Perfusing Spleen in the Absence of Added ACE. Table 2 shows

the change in cellular components of blood produced as a result of pulsatile perfusion of spleen with whole heparinized blood (previously perfused through lung) at a constant mean arterial pressure of 100 ± 10 mm. Hg. It will be seen that the number of circulating erythrocytes uniformly decrease at a slow rate (an average decrease of 2 per cent per hour), but that the lymphocyte count remains, on the average, relatively constant. Similar results were obtained whether glucose was or was not added to the perfusion system to maintain the initial glucose concentration.

Histological examination of spleens perfused for five hours as compared to non-perfused spleen revealed significant depletion of lymphocytes from the splenic red pulp, with partial replacement by agglutinated and conglutinated RBC and hemosiderin. The number and size of the Malpighian corpuscles and lymphoid follicles appeared
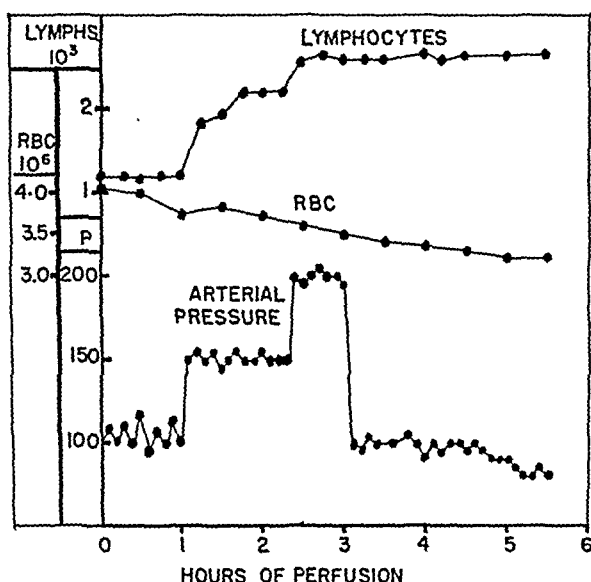
Fig. 3. The effect of altering the mean arterial pressure of a perfused spleen, upon the number of circulating lymphocytes and erythrocytes. All values in terms of cells per mm.^{3}. Arterial pressure in mm Hg.

somewhat smaller, although the significance of this change was diminished by the variability observed between different perfused splenic preparations. Further, the macrophages of the perfused spleens contained large amounts of debris, possibly of nuclear origin. These findings demonstrating loss of lymphocytes from the perfused spleens taken together with the fact that the number of lymphocytes in the circulation remains relatively constant suggests that the perfused spleen destroys circulating lymphocytes on one hand and adds splenic lymphocytes to the circulation on the other hand.

In the next experiments the effect of increasing splenic perfusion pressure upon the circulating blood cells was determined. In four ex-

TABLE 2. THE EFFECT OF SPLEEN PERFUSION, IN THE ABSENCE OF ADDED ACH, UPON THE CELLULAR ELEMENTS OF BLOOD

Exp. no.*	Blood sugar maintained§	Initial		Percentage change as result of spleen perfusion														
				30 min.		60 min.		90 min.		120 min.		180 min.		240 min.		300 min.	
		R†	L‡	R	L	R	L	R	L	R	L	R	L	R	L	R	L
1	No	4.00	1.90	-3.5	-4.2	-5.5	-8.9	-7.7	-12.6	-9.0	-16.8	-10.0	-19.0	-14.0	-19.0	-16.0	-19.0
2	No	4.06	3.92	-1	-5.4	-4.2	-9.3	-7.6	-15.8	-8.8	-15.5	-9.6	-11.2	-14.1	-10.5	-18.8	-10.0
3	No	4.01	1.77	—	—	-3.7	+1.7	—	—	-7.2	+7.3	-7.5	+4.5	-10.2	+13.0	-12.0	+11.0
4	Yes	4.32	1.35	-3.5	-10.4	-7.0	0	-7.2	-3.8	-7.3	-2.2	-8.3	-5.2	-10.0	-7.4	-11.0	-6.7
5	Yes	4.81	4.30	-1.6	-1.4	-0.8	-1.4	-1.2	-2.3	-3.1	-2.1	-2.5	-8.0	-3.0	-2.3	-4.0	-5.0
6	Yes	4.30	3.90	—	—	-3.5	-3.8	—	—	-7.0	-5.6	-6.5	-4.6	-7.5	-1.3	—	—
7	No	4.50	4.12	—	—	-2.5	-7.0	—	—	-5.6	-7.0	-1.1	-6.8	—	—	—	—
8	Yes	4.67	3.00	-2.0	+3.2	-2.0	+6.6	-1.7	+9.3	-1.5	+10.0	-3.0	+12.6	-3.7	+13.2	-6.0	+16.6

* Blood volume in these experiments varied from 110–130 cc.
† R is RBC count (×10⁶) per mm³.
‡ L is the absolute lymphocyte count (×10³) per mm³.
§ Blood sugar maintained by injection of 18–24 mg. glucose every 30 minutes into the pump.

periments, after a preliminary period of spleen perfusion at a mean
arterial pressure of 100 mm. Hg. the arterial pressure was increased
to 150–200 mm. Hg. Following the increase in arterial pressure, the
number of circulating lymphocytes uniformly rose while the level of
circulating erythrocytes was not affected. In three experiments, the
arterial pressure was then decreased to 100 mm. Hg. after the spleen
had discharged lymphocytes in response to the increase in the arterial
pressure. In no case during the subsequent two-hour period did the
level of circulating lymphocytes fall when the perfusion pressure was
decreased. Fig. 3 shows a typical experiment of this group, illus-

HOURS OF PERFUSION
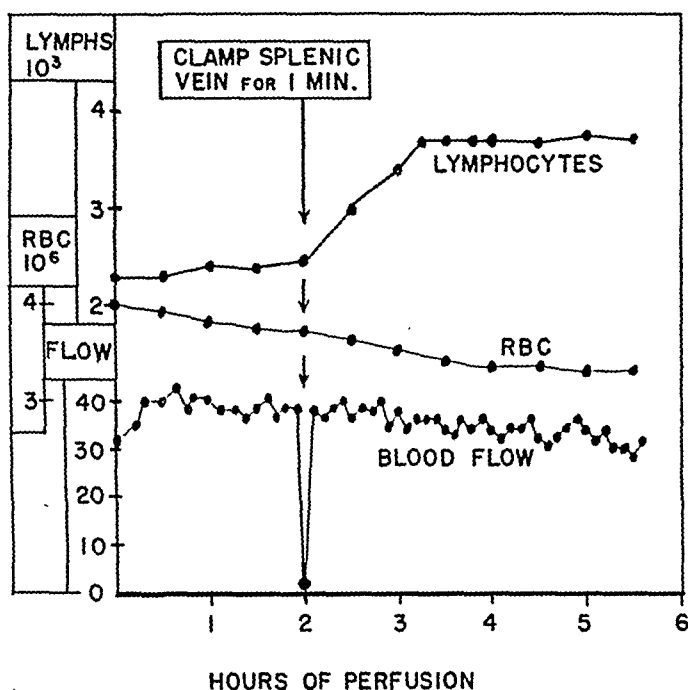
FIG. 4. The effect of temporary obstruction of the splenic vein of a perfused spleen
upon the number of circulating lymphocytes and erythrocytes. All values in terms of
cells per mm.³. Blood flow in cc. per min.

trating the effect of first increasing, then decreasing the perfusion
pressure of an isolated spleen upon the level of circulating lympho-
cytes.

To determine whether temporary increase of venous pressure
would likewise lead to lymphocyte discharge from spleen, in two ex-
periments the splenic vein of an isolated spleen was clamped for one
minute and then released, while the organ was being perfused at a
constant pressure. As a result of the venous obstruction, the splenic
volume markedly increased during the period of venous obstruction,
and then returned to normal following removal of the venous clamp.
In both experiments the level of circulating lymphocytes, following

temporary venous obstruction, increased to a maximal value which was then maintained, and here again, the level of circulating erythrocytes was not affected. Fig. 4 shows one of these experiments illustrating the effect of temporary venous obstruction of a perfused spleen upon the level of circulating lymphocytes.

These results demonstrate that under the influence of increased arterial or venous pressure, the rate of lymphocyte discharge from
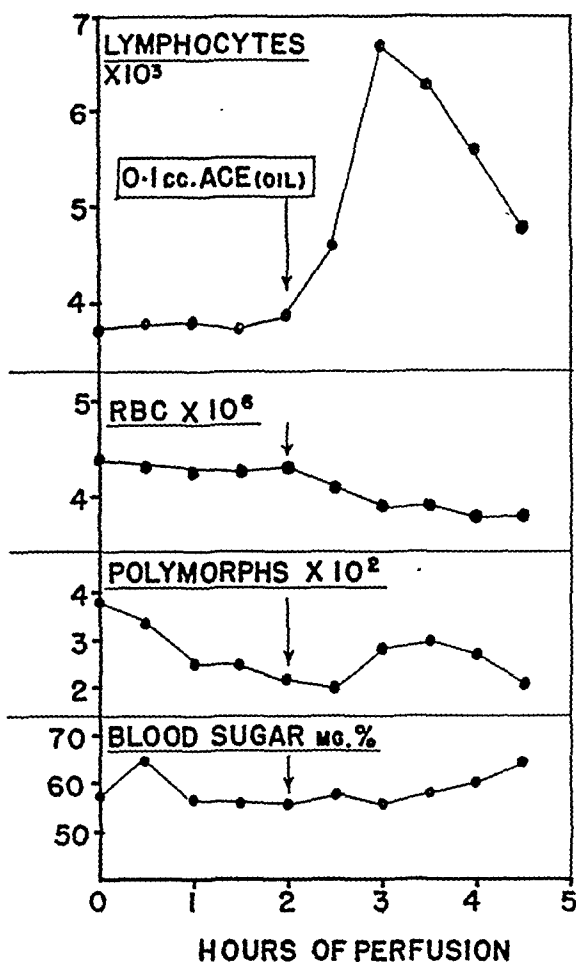
FIG. 5. The effect of adding ACE to a perfused spleen, upon the cellular components of blood. All values in terms of cells per mm.[3].

spleen is accelerated so that the level of circulating lyphocytes rises. During a 2-hour interval following the removal of the initiating stimulus, the level of circulating lymphocytes as the result of increased pressure was maintained and showed slight or no tendency to fall. It may therefore be inferred that the lymphocytes discharged by spleen because of increased pressure do not return to the spleen to be retained, nor does it appear that the initial rate of destruction of circulating lymphocytes secondarily increases after splenic lymphocytes are discharged.

Effect of Addition of ACE upon the Perfusion Spleen. In the next experiments the effect of adding ACE (oil or aq.) to the spleen perfused with a constant arterial pressure was studied. Fig. 5 shows the results of a representative experiment illustrating the effect of ACE (oil) upon the cellular elements in the perfusion medium. In this experiment the blood pressure varied between 90 and 100 mm. Hg., the blood sugar was maintained at approximately 60 mg. per cent by administration of glucose (15 mg. at 30 minute intervals); all extrasplenic blood vessels were ligated and after two hours of perfusion, 0.1 cc. of ACE (oil) was added to the system. It will be seen that following the addition of ACE, the number of circulating lymphocytes rapidly increased and then fell while the level of circulating erythrocytes or polymorphonuclear leucocytes was not affected.

Table 3 shows the composite results of 11 experiments on spleen perfused with constant mean pressure of 100 ± 10 mm. Hg. in which the effect of ACE upon the level of circulating lymphocytes was studied, in contrast to other experiments, to be mentioned later, where other agents in addition to ACE were tested. Since the levels of circulating erythrocytes or polymorphonuclear leucocytes were not influenced as the result of addition of ACE to the system, only the changes in circulating lymphocytes are shown. It will be seen that the addition of ACE to the perfused isolated spleen, was consistently followed by an increase in the number of circulating lymphocytes, amounting on the average to 1.60×10^8 lymphocytes. The splenic discharge of lymphocytes induced by ACE is uniformly observed despite the fact that the conditions employed in the individual experiments differed in several respects. From Table 3 it is apparent that the *in vitro* effect of ACE does not appear to be influenced *qualitatively* by the following factors:

(a) *Blood sugar level:* Compare experiments 2, 3, 5, 7, 8 to 1, 4, 6, 9, 10, 11.
(b) *Circulatory differences in the organ as the result of leaving certain non-splenic blood vessels open or closed:* Compare experiments 1–6 to 7–11.
(c) *Initial level of circulating lymphocytes:* Compare experiment 1 to experiment 6 or 8.
(d) *Nature of the vehicle:* Compare experiments 1, 2, 3, 4, 5, 8 to experiments 6, 7, 9, 10, 11. The conclusion that the vehicle for the ACE is not an important factor was substantiated further by observations that cottonseed oil or that small concentrations of chlorobutanol (the components of the oil vehicle for the lipo-adrenal extract) when tested alone or together had no influence on the isolated spleen.
(e) *Presence or absence of antibiotics:* Compare experiments 9 and 11 to the other experiments.

It should be emphasized that it is possible that any or all of the above factors may affect the *quantitative* aspects of the reaction wherein ACE produces lymphocyte discharge from the spleen. This possibility, however, remains for future investigation. At the present

TABLE 3. THE EFFECT OF ADRENAL CORTEX EXTRACT UPON THE ABSOLUTE LYMPHOCYTE COUNT OF THE PERFUSION BLOOD CIRCULATED THROUGH AN ISOLATED SPLEEN

Exp. no.	Wet wt. spleen (g.)	Blood vol. (cc.)	Blood sugar maintained*	Splenic circulation†	Antibiotics added	Lymphocyte count ($\times 10^3$) per mm³ after addition of spleen to pump							ACE administered‡		Lymphocyte count ($\times 10^3$) per mm³ after addition of ACE							Absolute lymphocyte discharge§ $\times 10^8$
						0 min.	30 min.	60 min.	90 min.	120 min.	150 min.	180 min.	Vehicle	Dose (cc.)	0 min.	15 min.	30 min.	45 min.	60 min.	90 min.	120 min.	
1	2.53	130	No	Open	Yes	4.12		3.83		3.66	3.82	3.80	Oil	0.6	3.80	4.75	5.95	6.44	7.00	6.45	6.15	4.16
2	2.12	132	Yes	Open	Yes	2.20	2.18	2.16		2.20	2.15	2.14	Oil	0.5	2.14	2.63	3.00	2.59	2.28	2.14		1.14
3	1.91	144	Yes	Open	Yes	2.28	2.50	2.83		2.78	2.84	2.90	Oil	0.5	2.90	2.98	3.56	3.30	3.40	3.21		0.95
4	3.85	138	No	Open	Yes	2.67	2.78	2.90	3.02	3.08			Oil	0.1	3.08		4.05	4.45	3.76		2.94	1.80
5	3.54	130	Yes	Open	Yes	3.68	3.80	3.80	3.74	3.93			Oil	0.1	3.93		4.53	6.68	6.40	6.35	6.10	3.58
6	2.54	110	No	Open	Yes	1.51	1.25	1.13	1.17	1.22			Oil	0.1	1.22		1.48	1.79	2.16	2.10		1.03
7	2.86	145	Yes	Closed	Yes	2.57	2.42	2.61	2.70	2.74			Aq.	1.0	2.74	3.23	3.48	3.01	2.79	2.70		1.07
8		117	Yes	Closed	Yes	1.52	1.51	1.54		1.39			Aq.	1.0	1.39	1.47	1.68	2.56	2.00	1.93		1.40
9	1.99	150	No	Closed	No	2.25	2.30	2.27	2.20	2.30			Oil	0.1	2.30	2.45	2.70	3.10	3.00	2.50		1.20
10	2.02	120	No	Closed	Yes	2.05	2.09	2.00	2.09	2.13			Aq.	1.0	2.09	2.63	2.58	2.60	2.48	2.15		0.65
11	2.54	140	No	Closed	No	2.71	2.70	2.73					Aq.	2.0	2.73		3.00		3.17	3.10	2.70	0.02

* Blood sugar maintained (70–100 mg. %) by injection of 18–24 mg. glucose every 30 minutes.
† Refers to whether splenic preparation had bleeders (open) or whether all non-splenic arterial vessels were ligated (closed).
‡ Hormone administered into pump, after the last sample in the control period was taken.
§ Splenic lymphocyte discharge induced by ACE estimated from the highest post-injection value, as compared to the initial lymphocyte level.

. time, mention is only made of the fact that from a *qualitative* point of view, these diverse factors do not appear to influence the *in vitro* effect of ACE upon spleen to produce lymphocyte discharge.

It will be seen from Table 3 that following the initial rise in circulating lymphocytes induced by addition of ACE to spleen, there is a tendency for the level of circulating lymphocytes to fall. Thus, in the eleven experiments shown in Table 3, the circulating lymphocytes return to the pre-ACE treatment value in four cases, in six experiments the return to the initial level is partial, while the increased level
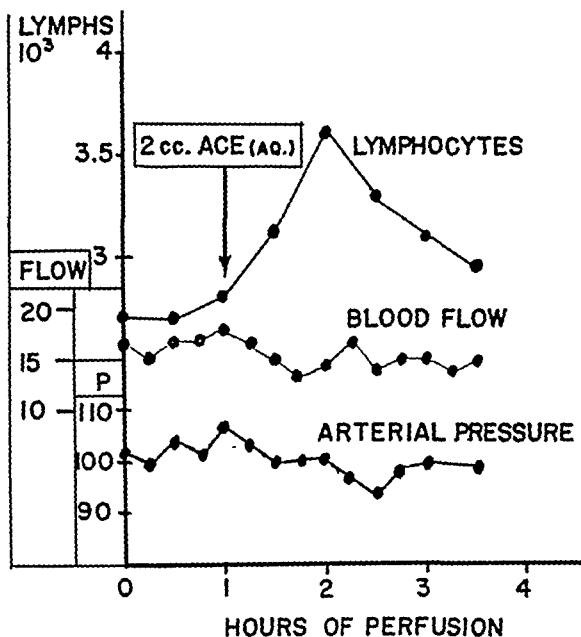
Fig. 6. Blood flow, arterial pressure, and circulating lymphocytes in a perfused spleen before and after addition of ACE. Lymphocytes per mm.³. Blood flow in cc. per · min., P-Arterial pressure in mm. Hg.

of circulating lymphocytes was maintained without a fall in only one of the eleven experiments.

To determine whether the secondary fall in circulating lymphocytes observed with ACE could be accounted for as the result of retention of circulating lymphocytes by the spleen, the splenic preparations perfused with ACE were sectioned and examined histologically. In this study the spleens were divided into three groups on the following basis: (a) a complete return of the lymphocyte level to the initial value, (b) only a partial return, and (c) no subsequent fall in the level of circulating lymphocytes. Histological examination revealed no significant difference between these three groups of spleens as regards the total number of lymphocytes in spleen. Thus, it would appear that the secondary fall of circulating lymphocytes observed

with ACE is not explicable on the basis that the decrease is due to
return and retention of circulating lymphocytes in the spleen. In all
groups, the histological picture was qualitatively similar to that pre-
viously described for spleens perfused in the absence of added ACE,
although there did appear to be a definite tendency for the ACE
treated spleens to show greater lymphocyte depletion.

Effect of ACE upon Blood Flow through Spleen. To determine
whether the effect of ACE upon the splenic discharge of lymphocytes
was associated with a change in blood flow through the spleen, meas-
urements of spleen blood flow were made in three experiments, before,
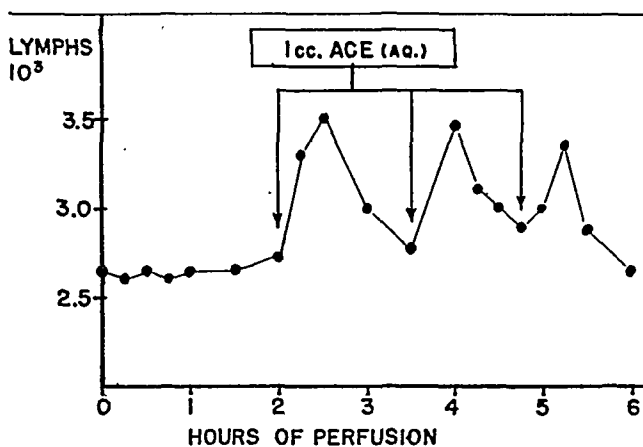
FIG. 7. The effect of repetitive addition of ACE upon the level of circulating lympho-
cytes (per mm.³) in an isolated perfused spleen.

during, and after ACE induced splenic discharge of lymphocytes. In
no instance did ACE produce a significant alteration of spleen blood
flow. Fig. 6 illustrates a typical experiment which demonstrates that
ACE induces the typical rise and fall of circulating lymphocytes with-
out significant alteration of splenic blood flow.

Repetitive Effect of ACE upon Spleen. The effect of repetitive
addition of ACE was determined in two experiments. In both cases,
it was observed that after the first dose of ACE had produced a rise
and fall of circulating lymphocytes, injection of another dose of ACE
produced a similar response. Fig. 7 illustrates an experiment wherein
three doses of ACE given separately produced three similar responses
of lymphocyte discharge from spleen, followed by subsequent removal.
or destruction, of circulating lymphocytes.

Effects of Other Agents upon the Isolated Spleen. Fig. 8 shows a repre-
sentative experiment of a group of three illustrating the ineffective-
ness of 1 mg. amounts of either estradiol dipropionate or desoxycorti-
costerone acetate to produce the splenic discharge response to an iso-
lated spleen preparation which has the capacity to respond to ACE
(oil) Fig. 9 shows a representative experiment of a group of three
illustrating the lack of effect of 100 mg. glucose or 0.1 mg. epinephrine
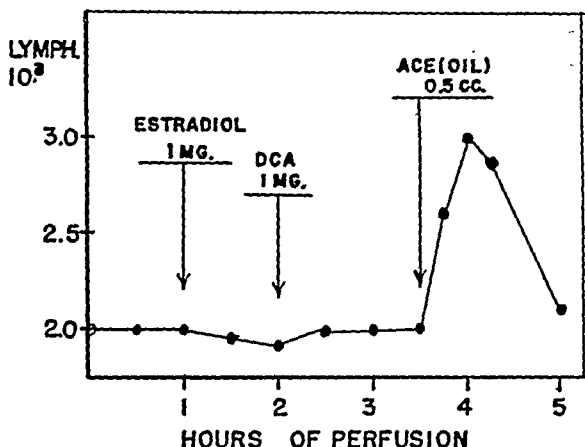
FIG. 8. The effect of adding estradiol dipropionate, desoxycorticosterone acetate, and ACE upon the level of circulating lymphocytes (per mm.³) in a perfused spleen system.

hydrochloride (1 cc. of 1:10,000) administered into the venous side of the pump upon the splenic discharge reaction. It will be seen that while neither agent influenced the level of circulating lymphocytes, addition of ACE (aq.) produced the usual discharge reaction in the same spleen preparation. In two additional experiments, 0.001 mg. epinephrine hydrochloride failed to induce splenic lymphocyte discharge.

The Effect of ACE upon other Isolated Organs. To determine whether the in vitro effect of ACE upon spleen was specific for that organ, the effect of ACE upon thymus lung, and liver were next studied.

In eight experiments where 0.5 cc. of ACE (oil) was added to perfused thymus preparations, it was uniformly observed that ACE never produced an increase in the number of circulating lymphocytes. In six of the experiments with thymus, no effect of ACE addition
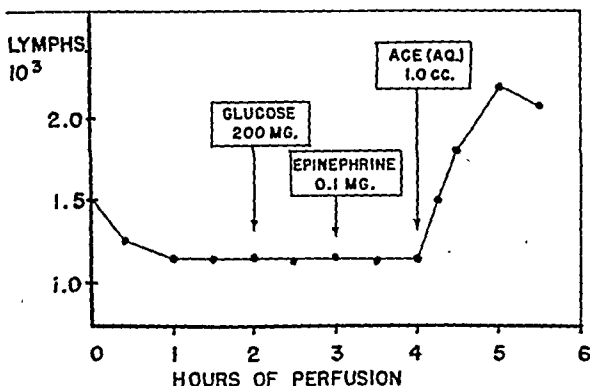
FIG. 9. The effect of adding glucose, epinephrine, and ACE upon the level of circulating lymphocytes (per mm.³) in a perfused spleen system.

upon the number of circulating lymphocytes was noted. Fig. 10a shows a representative experiment illustrating this lack of effect of ACE upon the level of circulating lymphocytes. However, in two experiments with thymus, it was observed, following the addition of ACE, that the circulating lymphocytes practically disappeared from the perfusion blood. Fig. 10b shows one of these experiments. No explanation for the differences in response following ACE addition to thymus is at hand, since the dosages of ACE employed, and all other known variables were similar in these experiments. It was noted, how-
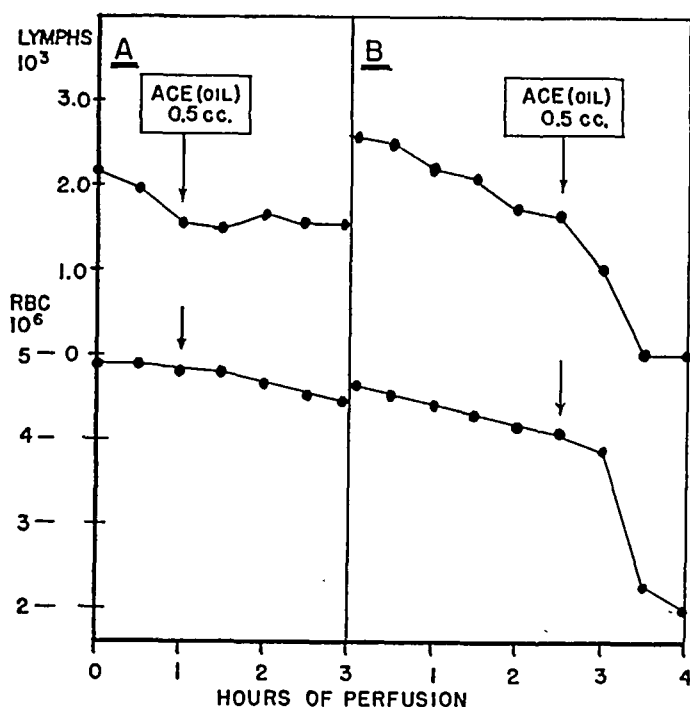
FIG. 10. The effect of adding ACE upon the cellular components of blood perfused through thymus gland. *10 A* represents the usual picture wherein ACE produces no significant effect. *10 B* represents one of two experiments wherein ACE produced a marked fall in circulating lymphocytes associated with a decrease in the number of circulating RBC. All values in terms of cells per mm.³.

ever that in both cases where ACE addition was associated with marked reduction of the number of circulating lymphocytes, a significant decrease of circulating erythrocytes also occurred. It may be mentioned that histological examination of the perfused thymus glands in these experiments did not clarify the differences in response noted.

While it is not possible to completely evaluate the action of ACE addition upon the perfused thymus, one conclusion seems definite: namely, that ACE added to perfused thymus does not produce a discharge of lymphocytes into the circulation, as evidenced by measurements of circulating lymphocytes.

Using lung in four experiments and liver in four experiments, it was consistently observed that the addition of 0.5 to 1.0 cc. ACE (oil) did not lead to any significant effect upon the number of circulating lymphocytes. Fig. 11 shows a representative experiment of lung and also a representative experiment on liver.

These negative results on the effect of ACE upon thymus, lung,
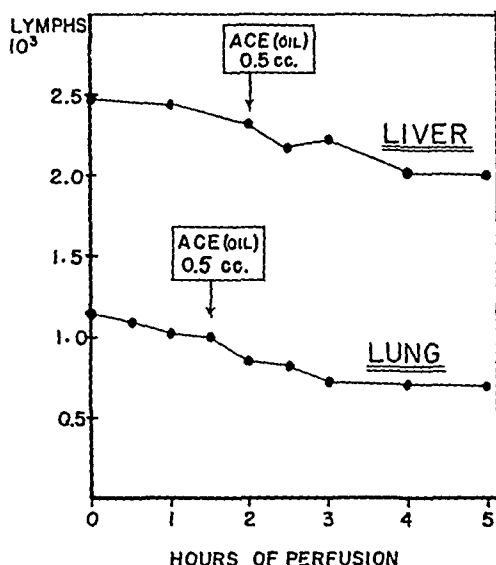
FIG. 11. The effect of adding ACE to lung and liver, upon the level of circulating lymphocytes (per mm.[3]) in the perfusion medium.

and liver suggest that the effect of ACE to produce lymphocyte discharge from tissues is specific for spleen.

DISCUSSION

These studies demonstrate that the addition of ACE to isolated rabbit spleen, perfused with heparinized blood at constant pressure, induces first a rise in the level of circulating lymphocytes, which secondarily tends to fall to the pre-treatment level. The rise in the number of circulating lymphocytes induced by ACE can be explained only on the basis of a splenic discharge of lymphocytes. This ACE induced splenic discharge phenomenon is rapid, occurring within 15 minutes, and reaches its maximum in 30 to 60 minutes.

In contrast to the rise in circulating lymphocytes, the reason for the secondary fall of lymphocyte level in the ACE treated spleen which occurs within a period of 2 hours of ACE administration, is less clear cut.

The secondary fall in lymphocyte level observed after ACE induced splenic discharge appears to be due to ACE, since splenic lymphocyte discharge produced as the results of increased arterial or venous pressure is not associated with a fall in the circulating lympho-

cyte level when the pressure stimulus is removed. It, therefore, appears that ACE induces the secondary fall in circulating lymphocytes either by facilitating (a) the return and retention or (b) the breakdown of lymphocytes in the isolated spleen. Histological examination of spleens perfused with ACE demonstrated consistent splenic lymphocyte depletion, independent of whether the secondary fall was absent, partial or complete. This histological finding thus strongly suggests that the secondary fall of lymphocytes observed with ACE is due to an increased breakdown of circulating lymphocytes by the perfused spleen. This evidence would seem to indicate that the well established *in vivo* action of ACE to increase the rate of lymphocyte dissolution in lymphoid structures (White and Dougherty, 1946) occurs under *in vitro* conditions of splenic perfusion. However, it should be emphasized that before it can be definitely stated that ACE accelerated the breakdown of lymphocytes under *in vitro* conditions of splenic perfusion, direct evidence of this process would be necessary.

The effect of ACE to induce *in vitro* splenic lymphocyte discharge could not be duplicated by estradiol dipropionate, desoxycorticosterone acetate, glucose or epinephrine, in the dosages tested. It is fully recognized that further work using a variety of steroids, as well as other agents, tested at varying dosage levels, will be necessary before the absolute specificity of the splenic discharge reaction to ACE will be delineated. However, these preliminary *in vitro* studies demonstrate that not all steroids produce the reaction, and that desoxycorticosterone acetate, which does not affect lymphoid structures or blood lymphocyte levels *in vivo* (White and Dougherty, 1946) likewise did not produce the splenic lymphocyte response.

Concerning the failure of epinephrine to induce discharge of splenic lymphocytes, several features of the experimental conditions employed *in vitro* must be considered before final conclusions are drawn. It should be noted that only two dosages of epinephrine were tested in a limited number of experiments; and that the epinephrine was administered into the venous side of the pump. The possibility therefore arises that the administered epinephrine was completely inactived in the perfusion blood before it could enter the spleen to act. Furthermore, it is known that certain end organ responses to epinephrine are qualitatively dependent upon the dosage of epinephrine (Grollman, 1936). It should be stressed that epinephrine in these experiments was tested solely to determine whether the effect of ACE upon the perfused spleen could be explained in terms of a possible epinephrine contamination of the extract. Our negative results with epinephrine have significance to the extent that they suggest that ACE action upon splenic discharge of lymphocytes cannot be explained on this basis. A detailed study of the effect of epinephrine upon *in vitro* splenic lymphocyte discharge using varied dosages, administered into the arterial circulation, remains for future investi-

gation, and may well reveal that epinephrine, as well as ACE, promotes this splenic reaction. Moreover, the fact that splenic lymphocyte discharge can be produced *in vitro* via increased perfusion pressure, suggests that epinephrine might produce splenic discharge of lymphocytes *in situ* via its pressor activity.

Regarding the specificity of ACE to induce lymphocyte discharge from other perfused tissues, it has been demonstrated that addition of ACE to perfused thymus, liver, or lung did not induce lymphocyte discharge. The finding that ACE did not discharge lymphocytes from a lymphoid structure such as thymus suggests that the ability of ACE to effect lymphocyte discharge may be related to the fact that splenic lymphocytes normally enter the blood directly, without first passing into the lymph stream, in contrast to the lymphocytes from other lymphoid structures.

What can be stated regarding the mechanism of ACE action to produce lymphocyte discharge from the isolated spleen? The results of this study throw light on this problem only in a negative way. Thus, the *in vitro* effect of ACE to produce splenic discharge can be produced without alteration of splenic blood flow or mean arterial pressure; nor has an obvious splenic contraction been visualized in association with this reaction. Whether the ACE effect to produce lymphocyte discharge from spleen is due to alteration in intrasplenic circulation, to splenic contraction, or to the release of lymphocytes from tissue elements to which they are fixed are questions which remain to be elucidated.

One aspect of our results upon the isolated rabbit spleen, not directly related to ACE action, merits discussion. It will have been noted that in all circumstances where splenic lymphocytes were discharged, whether by ACE at constant arterial pressure or as the result of increased splenic arterial or venous pressures, in no instance was there noted a coincident discharge of erythryocytes sufficient to increase the circulating red blood cell count. This finding, at first sight, appears to be in contradiction to the well established blood reservoir function of the spleen, whereby splenic contraction forces blood from this depot into the active circulation (Barcroft, 1926, 1930).

It should be recalled that the increase in circulating RBC observed *in vivo* (in some species) associated with splenic contraction is possible only because the blood stored in the spleen is richer in erythrocytes than the circulating blood. In our *in vitro* experiments with the isolated spleen, this situation probably does not exist for the following reasons: the splenic blood, originally present was first removed by the splenic contraction which occurred upon the death of the animal. Thus, the spleen inserted into the perfusion pump is in its contracted state with no concentrated blood. Upon initiation of circulation through the isolated spleen, the possibility of reconcentrating erythrocytes from the perfusion blood probably is limited due to the absence of regulated nervous control of splenic blood flow. *In situ*, blood

concentration in the spleen seems to be dependent upon rhythmic splenic contractions induced by regulated blood flow which causes the fluid elements of blood to be forced into lymphatic vessels while retaining the formed elements of blood in the red pulp (Hamilton, 1946). It, therefore, seems likely that the failure to observe erythrocyte discharge in our experiment is not due to the absence of this process but to the fact that it is not measurable because of lack of RBC concentration in the perfused spleen.

SUMMARY

The administration of adrenal cortex extract to the isolated rabbit spleen, perfused with whole blood at a constant mean pressure, induces a prompt discharge of splenic lymphocytes into the circulation. There is, secondarily, a tendency for the level of circulating lymphocytes in the perfusion medium to fall. The secondary decrease in lymphocyte count appears to be due to accelerated lymphocyte breakdown by spleen, in the presence of ACE. The activity of ACE to produce the *in vitro* reaction upon spleen is not associated with alteration of mean arterial pressure or changes in splenic flow, and can be demonstrated upon repetitive addition of ACE. The addition of ACE to perfused thymus, liver, or lung does not produce discharge of lymphocytes from these tissues; glucose, epinephrine, desoxycorticosterone acetate, and estradiol dipropionate in the doses tested, did not provoke lymphocyte discharge from spleen.

ACKNOWLEDGMENTS

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SPLENIC LYMPHOCYTE DISCHARGE INDUCED BY ADRENAL CORTICAL HORMONES UNDER IN VIVO CONDITIONS[1]

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IN A PRECEDING PAPER, it was demonstrated that adrenal cortex extract (ACE) produces lymphocyte discharge from the perfused rabbit spleen (Hechter, 1948). It was therefore of interest to determine whether ACE produces a similar reaction upon the spleen under *in vivo* conditions. In this connection, our attention was drawn to the fact that some stresses appear to be associated with an absolute lymphocytosis, instead of the lymphopenia which has been more commonly associated with increased adrenal cortical activity (cf. White and Dougherty, 1946). Thus, absolute lymphocytosis has been reported following: exercise in rats and mice (Harlow and Selye, 1937) and in man (cf. Garrey and Bryan, 1935), short-term (five minute) restraint in rats (Farris, 1938b), and so-called "emotional stimulation" in man (Farris, 1938a).

In view of our *in vitro* findings, it seemed possible that the lymphocytosis associated with these stress situations might in part result from a possible action of adrenal cortical hormone (ACH) upon the splenic lymphocyte discharge mechanism. To test this hypothesis, a preliminary investigation was undertaken to find a stress situation which would produce a significant degree of lymphocytosis. Since both exercise and so-called "emotional stimulation" in rats produce only slight rises in the absolute number of circulating lymphocytes, the effect of combining both stimuli by forcing animals to swim was studied. It was then observed that swim-stress in rats consistently produced a significant lymphocytosis. Accordingly, the effect of splenectomy and adrenalectomy upon this response was determined. In addition, the effect of ACE and desoxycorticosterone acetate (DCA) in adrenalectomized and adrenalectomized-splenectomized rats subjected to swim-stress was studied.

METHODS

Male albino rats, of the Sherman strain raised in this laboratory, weighing from 180 to 200 grams and fed a diet of Purina dog chow, were used in

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these studies. The adrenalectomized animals were prepared in the usual fash-ion, and after operation were given the stock diet plus 1 per cent NaCl in the drinking water. The animals were used for experimentation 2–3 days after adrenalectomy. Splenectomy was performed through a dorsal incision, 24 hours before experimentation, after which time these animals received no special treatment. For swim-stress, rats were placed in a bath-tub contain-ing water at 27°–28C. They were removed from the tub for blood sampling or injections. Blood samples were obtained from the tail; white and red blood cells were measured in the usual fashion; and differential leucocyte counts were made by counting 100 cells on the air-dried smears, stained with Wright's stain.
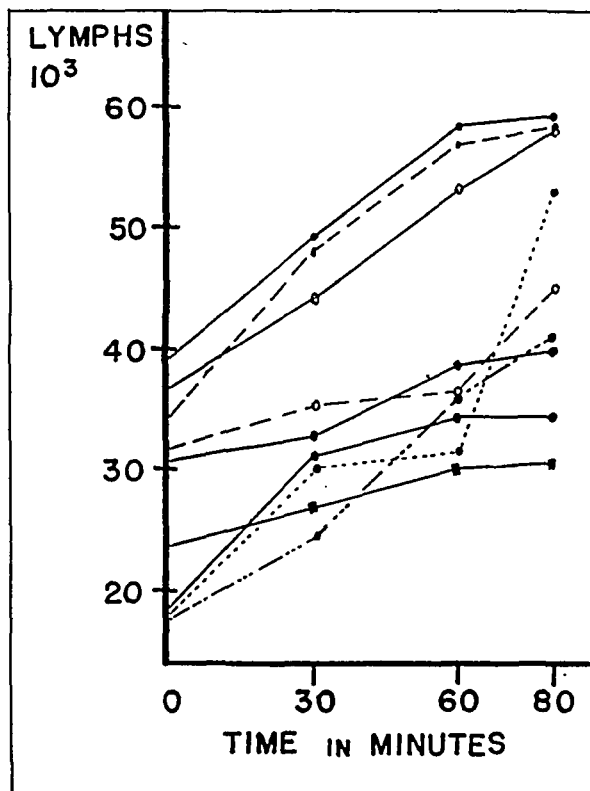
FIG. 1. The effect of forced swimming of normal rats upon the absolute lymphocyte level (per mm.³) of the blood. Each curve represents a single animal and it will be noted that absolute lymphocytosis regularly develops.

RESULTS

Fig. 1 shows the effect of swimming normal rats upon the absolute level of circulating lymphocytes. It will be seen that an absolute lymphocytosis uniformly develops. Table 1 illustrates the average re-sults in four rats subjected to swim-stress, in which circulating lym-phocytes and erythrocytes were simultaneously measured. It will be observed that the absolute lymphocytosis of swim-stress in normal rats is not due to hemoconcentration.

Table 2 illustrates the comparative results obtained upon sub-

jecting adrenalectomized, splenectomized, and normal rats to swim-stress. The results indicate that in normal rats there is a significant absolute lymphocytosis after 60 to 80 minutes of forced swimming. Following adrenalectomy, the lymphocytosis of swim-stress is abolished. Splenectomy significantly reduces the lymphocytosis response at 60 and 80 minutes (P values <0.01 as compared to normals). While the splenectomized rats subjected to swim-stress do exhibit some increase in circulating lymphocytes over their initial blood levels, this rise, it should be pointed out, is not statistically significant. Simul-

TABLE 1. EFFECT OF SWIMMING NORMAL RATS UPON THE PERCENTAGE CHANGE OF LYMPHOCYTES AS COMPARED TO ERYTHROCYTES

Rat no.		Percentage change	
		30 min.	60 min.
1	RBC	+21	+14
	L*	+38	+28
2	RBC	+15	+ 3
	L	+71	+85
3	RBC	+ 8	−15
	L	+58	+86
4	RBC	+24	+30
	L	+89	+80
Mean	RBC	+19	+ 8
	L	+64	+70

* Lymphocytes.

taneous counts of circulating lymphocytes and RBC in adrenalectomized or splenectomized rats forced to swim for 80 minutes revealed no significant changes from the initial values.

The next experiments were designed to test whether ACE or DCA could induce lymphocytosis in swimming adrenalectomized animals. In these experiments the rats were first forced to swim for 30 to 40 minutes, and then either 0.25 cc. of lipoadrenal cortex extract (Upjohn) or DCA (5.0 mg. per cc.) was injected intraperitoneally. After injection, the rats were returned to the water bath and forced to swim for another 60 minutes. Blood samples were obtained immediately before, and 30 to 60 minutes after the injection of ACE or DCA. Table 3 shows the results obtained. It will be observed that 60 minutes after ACE treatment swimming adrenalectomized rats develop a blood lymphocytosis. In contrast, DCA fails to induce lymphocytosis. Simultaneous red blood cell counts demonstrated that injection of ACE or DCA in swimming adrenalectomized rats was not associated with either hemo-dilution or hemo-concentration.

TABLE 2. THE EFFECT OF SWIM-STRESS UPON THE ABSOLUTE LYMPHOCYTE LEVEL IN NORMAL, SPLENECTOMIZED AND ADRENALECTOMIZED RATS

No. of rats	Type	0 min.	Period of forced swim											
			30 min.				60 min.				80 min.			
		L*	L	Diff.	P₁†	P₂‡	L	Diff.	P₁	P₂	L	Diff.	P₁	P₂
10	Normal	27.3 ±2.6§	35.7 ±3.0	% +31	0.05	—	41.8 ±3.8	% +53	<0.01	—	44.2 ±4.0	% +62	<0.01	—
11	Splenectomized	29.2 ±3.5	35.8 ±4.7	+23	0.2 to 0.3	0.1	36.6 ±4.3	+25	0.2	<0.01	36.1 ±4.9	+24	0.2 to 0.3	<0.01
12	Adrenalectomized	22.1 ±2.3	23.6 ±2.3	+7	>0.5	<0.01	22.0 ±2.1	+0.2	>0.5	<0.01	21.2 ±2.8	+4	>0.5	<0.01

* Is the absolute lymphocyte count ($\times 10^3$) per mm.^{3}.
† P₁ is the "p value," comparing mean lymphocyte levels after swim to pre-swim in the same group of rats.
‡ P₂ is the "p value," where the mean lymphocyte changes in swimming splenectomized or adrenalectomized rats is compared to the lymphocyte change in normal rats swum for the same periods of time.
§ Standard error of the mean.

To determine whether the lymphocytosis produced by ACE in swimming adrenalectomized rats was due to discharge of splenic lymphocytes into the circulation or to a possible mobilization of extrasplenic lymphocytes, the effect of ACE was tested in swimming adrenalectomized-splenectomized rats. In these experiments, 0.25 cc. ACE was injected intraperitoneally into the rats after they had been forced to swim for 30 to 40 minutes. These results are likewise shown in Table 5 from which it will be seen that ACE administered to swimming adrenalectomized-splenectomized rats does not produce lymphocytosis; indeed there is a tendency for ACE to produce lymphopenia under these conditions.

In an attempt to obtain direct evidence that splenic lymphocytes are discharged into the circulation under conditions of swim-stress,

TABLE 3. THE EFFECT OF ADRENOCORTICAL HORMONES UPON THE ABSOLUTE LYMPHOCYTE COUNT IN ADRENALECTOMIZED AND SPLENECTOMIZED-ADRENALECTOMIZED RATS, SUBJECTED TO SWIM STRESS

No. of rats	Type	Treat-ment	Time after injection						
			0 min.	30 min.			60 min.		
			L*	L	Diff.	P†	L	Diff.	P†
21	Adrenalectomized	ACE‡	24.7±1.69§	27.9±1.63	%+13	>0.05	31.2±1.69	%+26	<0.01
8	Adrenalectomized-Splenectomized	ACE‡	23.4±1.43	22.2±1.26	− 5	>0.5	2.06±1.03	−12	0.1–0.2
7	Adrenalectomized	DCA‖	25.0±2.73	28.2±4.0	+13	>0.5	21.8±3.26	−13	0.5

* L is the mean absolute lymphocyte count ($\times 10^3$) per mm.³.
† P is the "p value" where the mean change in lymphocytes after injection is compared to pre-injection value.
‡ 0.25 cc. lipo-adrenal extract injected intraperitoneally.
§ Standard error of the mean.
‖ 0.25 cc. of desoxycorticosterone acetate (5 mg./cc.) administered intraperitoneally.

the number of lymphocytes extractable from spleen were measured in normal, unstressed rats and matched rats which were forced to swim for 60 minutes. To check the hypothesis that splenic discharge fails to occur in the absence of the adrenals, similar experiments were made comparing the number of splenic lymphocytes in non-stressed adrenalectomized rats with those of adrenalectomized rats forced to swim for 60 minutes. In these experiments, the spleen was removed from etherized rats, weighed, ground to a mash with saline in a mortar, and then transferred to a volumetric flask. To destroy the RBC present in the extract, 10 cc. of 0.1N HCl was added, the volume was adjusted to 100 cc., and WBC counts were made in the usual manner. It was assumed that all of the WBC were lymphocytes since smears made on the mortar mash revealed that practically all of the leucocytes in the spleen extracts were lymphocytes. Fig. 2 shows the effect of swimming normal and adrenalectomized rats upon the total number of lymphocytes extractable from the spleen. It will be seen that normal animals, subjected to swim-stress, exhibit an average decrease of 27 per cent in the number of splenic lymphocytes from the initial unstressed value, while swim-stressed adrenalectomized rats show only

an average decrease of 7 per cent. While these findings tend to agree with the view that splenic discharge occurs in normal animals, but not in adrenalectomized rats, subjected to swim-stress it should be emphasized that the results are not statistically significant. Whether the variability in the results observed is due to the method employed or is inherent in the nature of the experiment, remains to be eluci-
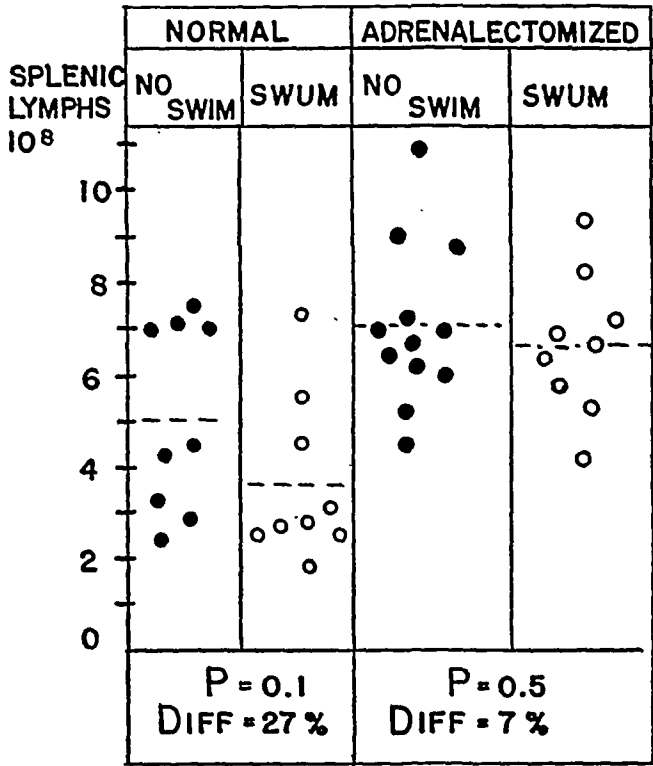
FIG. 2. The absolute number of lymphocytes extractable from spleens of normal and adrenalectomized rats after a 60-minute swim, as compared to non-swum normal and adrenalectomized animals. Each dot or circle represents a single animal. The dotted lines represent the mean values for the various groups.

dated. It may be mentioned, as an incidental finding, that there appears to be a greater number of lymphocytes in the spleens of adrenalectomized rats as compared to normals.

DISCUSSION

These studies strongly suggest that endogenous adrenal cortical hormones induce splenic lymphocyte discharge in rats subjected to swim-stress. Thus, the absolute lymphocytosis associated with forced swimming can be significantly reduced either by splenectomy or adrenalectomy, and can be re-induced in swum-adrenalectomized rats by the administration of ACE only if the spleen is intact. Taken together, with the *in vitro* studies described in the previous paper, these findings provide strong evidence for the view that the control of

splenic lymphocyte discharge by adrenal cortical hormones is a physiological mechanism.

The finding that DCA, in contrast to ACE, cannot induce lymphocytosis in swum-adrenalectomized rats is likewise in agreement with the perfusion studies wherein DCA was ineffective. These results therefore suggest that the splenic lymphocyte reaction which has been discussed is under control of those hormonal corticosteroids which possess an oxygen atom at position 11 of the steroid nucleus, e.g. the so-called "sugar-active" cortical hormones. It should be emphasized that the same ACE which induces lymphocytosis in swimming adrenalectomized rats exhibits a potent lymphopenic activity when tested in adrenalectomized rats or mice.

An estimate of the number of lymphocytes discharged from the spleen of swimming rats by administration of ACE may be made by comparison of the lymphocytosis response produced by ACE in swum adrenalectomized and adrenalectomized-splenectomized rats. On the assumption that the rates of peripheral removal of lymphocytes and of extra-splenic lymphocyte delivery of lymphocytes to the blood are the same in both groups, it may be calculated that, on the average, the absolute lymphocyte level is increased 32 per cent by discharge of splenic lymphocytes. Assuming a blood volume of 18 cc. for 200 gram salt-treated adrenalectomized rats (Hechter, 1945) this would represent an average discharge of 1.44×10^8 lymphocytes from rat spleen. This value is approximately 30 per cent of the total number of lymphocytes which we have found to be extractable from the spleens of normal non-stressed rats.

It is of interest to compare the calculated estimates of splenic discharge induced by ACE under *in vitro* conditions from rabbit spleen with the above values. From the perfusion studies described in the preceding paper, it was calculated that approximately 1.60×10^8 lymphocytes are discharged from the average rabbit spleen. Discharge of this number of lymphocytes in a 3 kgm. rabbit (assuming a blood volume of 430 cc. and an initial lymphocyte count of 7000 per cmm.) would increase the blood lymphocyte level only about 5 per cent. In general, it would appear that the importance of ACE-induced splenic lymphocyte discharge upon the blood lymphocyte level among various species would depend to a significant degree upon the ratio of the total number of splenic lymphocytes to the circulating blood volume or, less precisely, to spleen weight/body weight. Species such as the rabbit, where the ratio is low (compared to the rat, for example) should not be expected to exhibit striking alterations in circulating lymphocyte levels as the result of the splenic reaction.

The physiological significance of the splenic discharge reaction, its participation in stresses other than swimming, and its relationship to the well established effect of ACH to produce blood lymphopenia (White and Dougherty, 1946) remains to be evaluated by future work.

It is apparent that the lymphopenic activity of ACH would tend to counteract the effect of the splenic reaction upon the circulating lymphocyte level and that the resultant effect would depend upon the magnitude, latent period and duration of these opposing actions.

SUMMARY

The absolute lymphocytosis produced by swim-stress in normal rats can be significantly decreased either by adrenalectomy or splenectomy. Administration of ACE to swimming adrenalectomized rats produces lymphocytosis; desoxycorticosterone acetate administered under these conditions does not produce lymphocytosis. Administration of ACE to adrenalectomized-splenectomized rats subjected to swim-stress does not produce lymphocytosis. These findings provide strong evidence for the view that ACH induces splenic lymphocyte discharge into the circulation.

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THE EFFECT OF REMOVING VARIOUS ENDOCRINE GLANDS ON THE HAIR CYCLES OF BLACK RATS[1]

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THE MANNER in which Norway rats grow their fur and replace it throughout life has been described in a previous paper (Dieke, 1947). It was shown there that in this species the hair follicles near the midventer are the first to become active, then those on the sides and finally those on the head and the rump, so that a complete new coat is acquired in a wave-like fashion, with only the follicles in certain circumscribed areas being active at any given time. Each complete wave of hair growth is called a hair cycle. In black rats the pigment which will color the hair can be seen in the skin just before the hair shafts emerge, which makes it easy to recognize and follow the active phase of each cycle.

In rats of this laboratory the first hair cycle begins several days after birth and is complete at about three weeks of age; the second begins at about one month and is usually completed within another month, while the third cycle begins at about 50 days and takes approximately 2½ months for completion. Subsequent cycles start at increasingly greater intervals and show progressively greater delay in completion; for this reason a half or full grown rat usually has several cycles in progress simultaneously.

A preliminary study has now been made to see whether these hair cycles are under endocrine control. Observations have been made, over periods up to 10 months, on rats surgically deprived of either adrenals, gonads, thyroid (and parathyroids), or hypophysis, with normal (unoperated) litter-mates serving as controls. There were male and female rats in each group.

Except for the adrenalectomized rats, which had access to a bottle of 3 per cent salt solution in addition to their regular food and water, all rats were treated identically, receiving the same standard rations customarily fed in this laboratory and drinking tap water. They were housed in individual cages in a well-ventilated inside room subject to no sharp fluctuations of temperature.

The operations were performed when the rats were between 34 and 38 days old. Each rat was then examined carefully twice a week thereafter for fur and skin changes, and the fur was clipped off from one side of the body at weekly intervals, or whenever it had grown sufficiently to obscure the skin pigmentation patterns. The fur on the other side of the body was left undisturbed throughout; it showed no greying or other deviation from normal except that the texture remained soft and juvenile in the hypophysectomized animals.

Completeness of operation was checked in each case at autopsy.

RESULTS

The 2nd hair cycle began at an average age of 30 days (range 28–32 days), before any operations had been performed. Table 1 shows how the various operations acted to delay or advance subsequent cycles.

TABLE 1. AVERAGE AGE. OF RATS IN DAYS AT START OF
HAIR CYCLES 3 THROUGH 7
(Operations performed at 34 to 38 days of age. Patterns were "normal" after adrenalectomy and gonadectomy, "reversed" after thyroidectomy and hypophysectomy.)

Operation	No. rats	Cycle				
		3rd	4th	5th	6th	7th
None (controls)	2 ♂, 4 ♀	52	81	113	(139–211)	(198–255)
Adrenalectomy	2 ♂, 3 ♀	50	68	93	115	142
Gonadectomy	1 ♂, 3 ♀	57	88	130	205	—
Thyroidectomy	2 ♂, 4 ♀	58	125	200	—	—
Hypophysectomy*	1 ♂, 4 ♀	82	118	179	226	268

* Values given after 3rd cycle are for 1 rat only.

For adrenalectomized rats the 3rd cycle began 2 days sooner than the controls. Subsequent cycles began progressively earlier than normal, so that these rats were starting their 6th cycle at an age (16 weeks) when the control rats had only just begun their 5th. In one case the 12th cycle started before the 7th of a control rat. The rapidity with which the cycles succeeded each other in the adrenalectomized rats, and their coherence compared to later cycles in normal rats, produced the striped effect illustrated in Fig. 1B, where 5 distinct waves of skin pigment appear corresponding to the active growth regions of cycles 7 (in the lumbar region) through 11 (on the belly). Fig. 1A shows for comparison a litter-mate control rat of the same sex, photographed at the same time; here a remnant of cycle 7·appears on the mid-dorsum and fragments of 8 lower on the side.

The gonadectomized rats had hair cycles essentially similar in appearance to those of the controls, and appearing with only slight delay, as may be seen in Table 1. The only marked deviation from a normal appearance of the skin in these rats was in the distribution of the superficial spots of oxidized lipoid material normally found on the backs of male but not of female rats in this colony (Dieke, 1947).

RAT 5 AJ
B♂—b. 6-4-46
UNTREATED

DATE	AGE
2-10-47	251 DAYS

RAT 6 AJ
B♂—b 6-4-46
ADRENALECTOMY
AT 39 DAYS

DATE	AGE
2-10-47	251 DAYS

RAT G7AJ
B♂—b. 6-16-46
HYPOPHYSECTOMY
AT 34 DAYS

DATE	AGE
2-10-47	239 DAYS
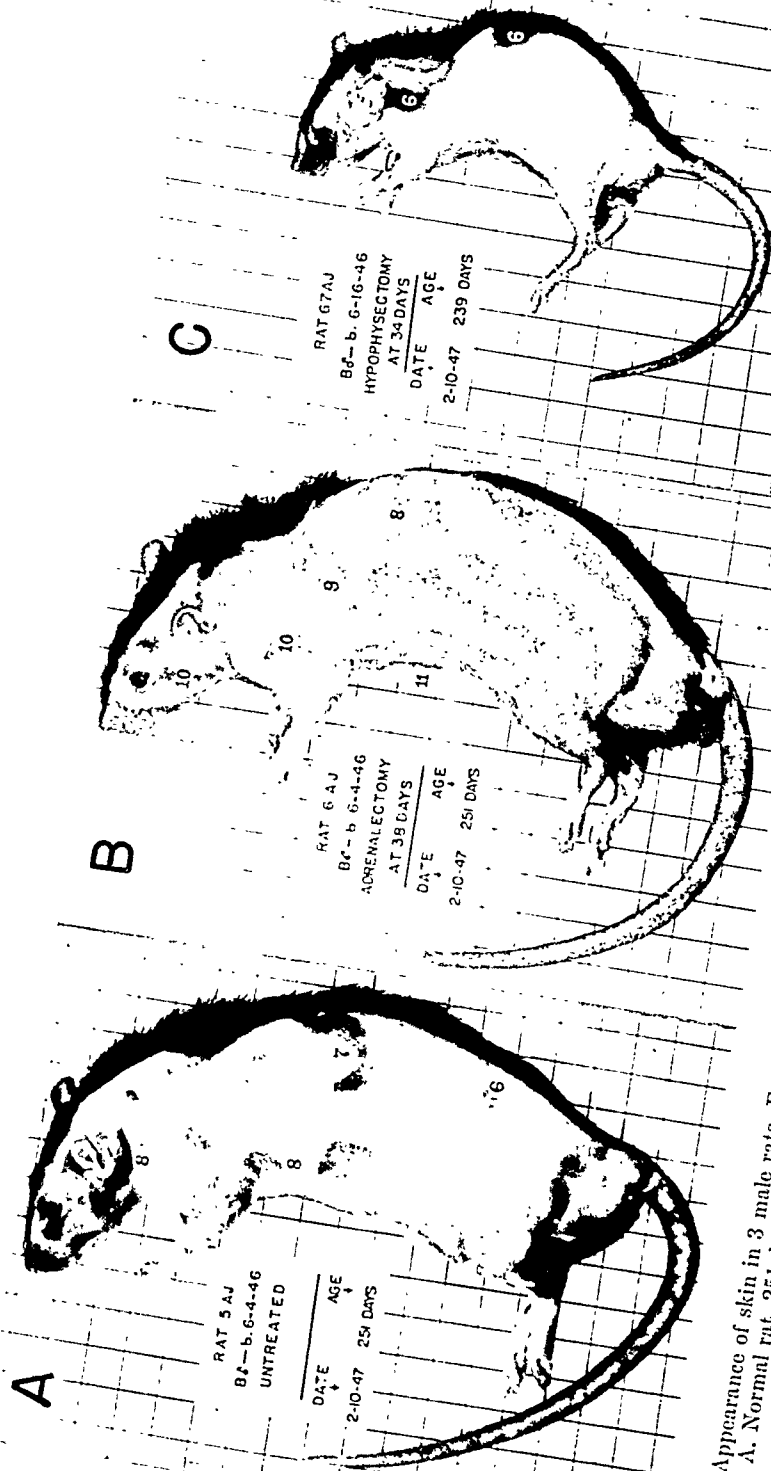
FIG. 1. Appearance of skin in 3 male rats. Fur clipped off in each case just before photographing. Numbers have been added to identify the hair cycles. A. Normal rat, 251 days old; B. Adrenalectomized litter mate to normal rat, also 251 days old; C. Hypophysectomized rat, 239 days old.

The ovariectomized rats showed considerable numbers of these spots, while in the one castrated male they were far fewer than normal, although not entirely absent.

The thyroidectomized rats not only showed great delay in hair replacement, but also had markedly abnormal patterns in those cases judged to have undergone successful operations. The first evidence of a new cycle (the 3rd) after operation did not appear on the belly but rather in a spot on the back of the neck, which then expanded in all directions, so that active growth progressed in a direction exactly contrary to the normal. Two of these rats died at 136 and 189 days of age, without ever starting anything recognizable as a 4th hair cycle. Two others ran through the 4th and 5th cycles, always starting them in the same spot on the back, while the remaining two had greatly delayed cycles but of the normal type. A small remnant of thyroid tissue was found in each of these last 2 rats when they were sacrificed at 9 months of age.

The hypophysectomized rats for the most part did not thrive as well as the others, presumably because removal of the fur was more difficult for them to endure. All but 1 died when between 75 and 135 days of age, having started but never completed the 3rd hair growth cycle. The 1 surviving hypophysectomized rat (the male) made a good adjustment and was in excellent health when sacrificed for autopsy at 337 days of age. (No remnants of hypophyseal tissue were found.) In this rat the hair cycles started, as in the thryoidectomized rats, at a spot on the back of the neck, which then spread out. Occasionally a second spot appeared somewhat later in the mid-dorsal region, and growth from it likewise proceeded outward. This rat's 6th hair cycle is illustrated in Fig. 1C.

The differences noted between the hair cycles of the operated and control rats were thus of 2 types: changes in the speed with which normally progressing cycles succeeded each other and reached completion, and more fundamental changes in the replacement patterns themselves. The former occurred to a greater or less degree after all the operations, while the latter were found only in thyroidectomized and hypophysectomized rats.

Previous workers have demonstrated alterations in the rate of hair growth which are related to endocrine factors. For instance, Chang (1926) found that thyroidectomy retards hair growth, Snow and Whitehead (1935) observed that hypophysectomized rats take longer to grow a full coat of hair, and Butcher (1937) showed that adrenalectomy stimulates hair growth. We have, however, found no published report which mentions the fundamentally different patterns occurring after removal of thyroid or hypophysis.

SUMMARY

Thyroidectomized or hypophysectomized rats not only showed delay in starting and completing hair-growth cycles, but also had

cycles which proceeded from a different starting point in a direction contrary to the normal. Adrenalectomized rats had greatly accelerated cycles which did not become fragmentary but retained their coherence with advancing age. Gonadectomy had no marked effect on the rats' hair cycles.

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URETHANE-INDUCED LYMPHOPENIA IN NORMAL AND ADRENALECTOMIZED RATS

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A DECREASE in the circulating lymphocytes as well as other physiological changes in normal rats after urethane injections was reported by Hawkins and Murphy (1925). The palliative treatment of human and experimental lymphatic and myelogenous leukemia with urethane has been reported by several investigators (Patterson, Thomas, Haddow and Watkinson, 1946; Haddow and Saxton, 1946; Webster, 1947; Kirschbaum and Lu, 1947; Engstrom, Kirschbaum and Mixer, 1947). Murphy and Strum (1946) reported that urethane treatment of rats with transplanted lymphatic leukemia resulted in a decrease in the white blood cell count and the lymphocytes. Since these investigators also observed adrenal gland hypertrophy, they suggested that this might be of significance in causing the depression of the lymphocytes in their leukemic rats. In this laboratory we had observed enlarged adrenal glands in normal rats after urethanization during the course of an investigation of some of the physiological effects of urethane (unpublished data). Since a lymphopenia can be induced in normal rats with certain of the adrenal cortex steroids (Dougherty and White, 1944), the occurrence of hypertrophied adrenals in urethanized leukemic and normal rats was suggestive of a mode of action of urethane on white blood cells via the adrenal mechanism. The experiments reported here were designed to test this hypothesis. It is the object of this paper to show that the adrenal has no role in the leucopenia and lymphopenia which follows urethanization of the normal rat.

MATERIALS AND METHODS

Adult male normal rats of the Sprague-Dawley strain were used in these studies. The rats were fed Purina Chow and tap water ad. lib. In the case of adrenalectomized rats the tap water was replaced by 1% NaCl solution. The conditions of the experimental procedures were kept as constant as possible throughout the experiments.

Total and differential leucocyte determinations were done by standard procedures on blood obtained from the tails of the rats. The differential leucocyte values were computed after counting at least one hundred white cells on blood smears stained with Wright's blood stain. A 10% urethane solution was used in all the experiments. All urethane injections were given

intraperitoneally at a dose level of 750 mg. per Kg. of body weight on Monday, Wednesday, and Friday. Blood samples for leucocyte counts were obtained from the rats at weekly intervals while they were still under the narcotizing effects of a urethane injection. Evipal (10.0 mg. per 100 gm. of body weight) was given intraperitoneally as an anesthetic when blood samples were required from non-urethanized rats or when urethane for anesthetic use was contra-indicated by the experimental procedures. Information regarding other substances administered in the course of these experiments will be described in the section containing the data.

RESULTS

The effect of urethane in normal intact and adrenalectomized rats on total circulating leucocytes is shown in Table 1. The average white

TABLE 1. ALTERATION OF TOTAL CIRCULATING WHITE BLOOD
CELLS AFTER URETHANE IN NORMAL RATS

Animal used	WBC per c.mm.	Per cent change
Normal Intact Rats: Controls (60)*	21,671 ± 934†	−28.4
Urethane (21)	15,512 ±1,630 P = <0.01	
Adrenalectomized: Controls (17)	22,370 ±1,889	−29.6
Urethane (7)	15,743 ±1,181 P = <0.01	

* Number in ( ) is the number of rats in the experiment.
† S.E. of the Mean.
10% urethane given I.P. tri-weekly at a dose level of 750 mg. per kg. for a total of eight injections.

blood cell value of 60 untreated normal rats is compared with the average value obtained from 21 of these rats after a course of eight intraperitoneal injections of a 10% urethane solution. The average white blood cell value of seventeen untreated adrenalectomized rats is compared with the average value obtained from seven adrenalectomized rats treated with urethane in a similar manner as the intact rats. Urethane treatment of the adrenalectomized rats was started two weeks after the operation. Only those adrenalectomized rats which were found free of adrenal bodies at autopsy were considered in these data. Inspection of Table 1 shows that the average circulating leucocyte value was approximately the same for the urethanized intact and adrenalectomized rats; and that this average value represents an approximate 30% depression from the respective control average figure. It is evident from these data that urethane treatment of normal rats resulted in a leucopenia which was not mediated through the adrenal mechanism.

Evidence of the changed leucocyte composition of the blood of the normal rat after urethane is shown in Table 2. The average values of

the total white blood cell and the absolute lymphocyte and polymor-phonuclear leucocytes per c.mm. of blood are given in this table for seventeen intact and eight adrenalectomized rats before and after urethanization. The averages for the control adrenalectomized rats were computed from blood samples taken two weeks after the opera-tion. The urethane treatment was the same for both groups—eight intraperitoneal injections of a 10% solution given tri-weekly at a dose level of 750 mg. per Kg. of body weight. It is evident from these data that the leucopenia which followed the prescribed course of urethani-zation was the result of an absolute lymphopenia which was of similar magnitude in the norml and the adrenalectomized rats. The absolute number of polymorphonuclear leucocytes was unaffected in these urethanized rats.

TABLE 2. THE LYMPHOPENIC EFFECT OF URETHANE IN NORMAL RATS

Animal used	Lymphocytes absolute number per c.mm.	Polymorphonuclear absolute number per c.mm.	WBC per c.mm.
Normal Intact Rats: Controls (17)* Urethane (17)	18,824 ±2,512† 9,407 ± 106 P = <0.01	4,469 ± 875 5,708 ±1,340 P =0.5	23,371 15,524
Adrenalectomized: Controls (8) Urethane (8)	14,989 ±1,173 10,396 ± 765 P = <0.01	6,379 ± 530 4,042 ± 800 P = <0.05	21,388 14,519

* Number in ( ) is the number of rats in the experiment.
† S.E. of the Mean.
10% urethane given I.P. tri-weekly at a dose level of 750 mg. per kg. for a total of eight injections.

The change in the composition of the circulating white blood cells after urethane is essentially different from that following the secretion of certain adrenal cortex hormones (Dougherty and White, 1944). These investigators have shown that the lymphopenia which results from adrenal stimulation is at a maximum approximately six hours after the injection of anterior pituitary adrenocorticotrophic hormone and then gradually rises to normal value by the twentieth hour. The lymphopenia which follows urethanization does not appear in the procedure which we followed until after the fifth or sixth injection of urethane. The absolute value of the lymphopenia after urethanization then remains with further injections at approximately 9000 lympho-cytes per c.mm. Dougherty and White had also shown that a poly-morphonulear leucocytosis was induced shortly after adrenal stimu-lation with A.C.T.H. and returned to normal absolute value by the ninth hour after injection. Since urethanization did not apparently affect the polymorphonuclear leucocyte picture in the blood of these rats, it was of interest to see whether this would still be so after ad-

renal cortical stimulation of previously urethanized rats.

An inspection of Table 3 shows that the average absolute number of polymorphonuclear leucocytes can be significantly increased in urethanized rats by adrenal cortical stimulation. The average white blood cell and absolute number of lymphocytes and polymorphonuclear leucocytes per c.mm. of blood were determined for seven normal intact rats and eight urethanized rats before and after a series of consecutive injections of an adrenaline solution. The adrenaline was injected according to a procedure recommended by Long and Fry (1945) to attain adrenal cortex stimulation. The adrenaline used was a 0.02%

TABLE 3. THE NEUTROPHILIA-INCITING EFFECT OF ADRENALINE
IN URETHANIZED NORMAL RATS

Animal used	Lymphocytes absolute member per c.mm.	Polymorphonuclear leucocytes absolute number per c.mm.	WBC per c.mm.
Normal Intact Rats: Controls (7)* Adrenaline (7)‡	13,125 ±1,084† 15,930 ±1,412 P = <0.2	2,888 ± 601 3,526 ±30 P =0.3	16,429 19,714
Normal Intact Rats: Urethane (8) Adrenaline (8)	8,202 ± 446 8,644 ± 743 P = <0.5	2,713 ± 376 9,456 ±2,488 P =0.02	11,225 18,788

* Number in ( ) is the number of rats in the experiment.
† S.E. of the Mean.
‡ Adrenaline given sub-Q at a dose level of 0.02 mg. per 100 gm. of body weight for four successive hourly intervals.
10% urethane given I.P. tri-weekly at a dose level of 750 mg. per kg. for a total of eight injections.

solution and was injected subcutaneously at four successive hourly intervals at a dose level of 0.02 mg. per 100 gm. of body weight. The adrenaline was administered to the urethane treated rats on the day after the eighth urethane injection. The data of Table 3 show that the intact non-urethanized rats were unaffected by the adrenaline injections insofar as their white blood cell composition was concerned. However, the urethanized rats had a completely distorted leucotyte picture twenty hours after the adrenaline injections. These rats had a polymorphonuclear leucocytosis along with a previously existing urethane-induced lymphopenia. It is apparent that urethane's effect on the white blood cells is not restricted to the lymphocytes; and that urethane is somehow related to the adrenal cortex mechanism as shown by its effect on the polymorphonuclear leucocytes.

DISCUSSION

Three general modes of urethane action are extant in the literature to explain its physiological effects on blood cells and tissues in general. Webster (1947) suggests that urethane induces a factor to which leukemic cells are especially sensitive. Kirschbaum and Lu (1947) em-

phasized their observation that there was an increased number of mature circulating white blood cells after urethane treatment of leukemic animals and suggested that these cells were more readily discarded by the leukemic animal. The second possible mode of action of urethane was that inferred from the hypothesis of Murphy and Strum (1946) that the hypertrophied adrenals secreted an increased quantity of a lymphocytolytic factor. This would place urethane in the large category of substances whose general effects upon the organism is explained by the General Adaptation Syndrome (Selye, 1946). Since the adrenal cortex is fundamental in inducing those changes from the physiological norm which are seen in the Adaptation Syndrome, the data presented in Tables 1 and 2 on the adrenalectomized rats makes this explanation untenable. The enlarged adrenal glands recorded by Murphy and Strum and by the authors in leukemic rats and normal rats, respectively, might be an alarm reaction to urethane secondarily but directly the result of the liberation of proteolytic products by this compound (Selye, 1946). This third possible mode of action of urethane is supported by the observations of several investigators— Warburg (1910) had shown that phenyl urethane in small amounts arrested mitosis and cell division in fertilized eggs of the sea urchin; Guyer and Claus (1947) reported the practical abolition of mitosis in the cornea of the rat; Engstrom, Kirschbaum and Mixer (1947) reported a decrease in the size of the spleen and lymph nodes of urethanized animals; and Kirschbaum and Lu (1947) observed a decrease in the number of mitotic figures in marrow myeloid cells of mice with myelogenous leukemia after urethane injections. Since we have shown that a lymphopenia can be induced with urethane in normal adrenalectomized rats and recently Lu and Kirschbaum (1947) reported that the adrenal mechanism was not involved in the effects of urethane in leukemic mice, it appears that urethane behaves as a mitotic poison insofar as it affects the lymphocytes. The alteration of the polymorphonuclear leucocyte picture after adrenal stimulation of urethanized normal rats cannot be explained, but it is under investigation.

SUMMARY

A leucopenia and an absolute lymphopenia of similar magnitude was induced in normal intact and normal adrenalectomized rats following the intraperitoneal administration of a 10% urethane solution. The evidence presented here showed that the adrenal glands were not involved in these changes in the blood picture. A possible mode of urethane action was discussed.

A relation of urethane with the adrenal mechanism was suggested by a polymorpho-leucocytosis which was induced in urethanized rats twenty hours after adrenal cortical stimulation.

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NOTES AND COMMENTS

GOITEROUS CHICKS FROM THYROPROTEIN-FED HENS

It has been shown that goiterous dams, or dams fed goiterogenic diets produce offspring with enlarged thyroids (Kalkus, 1920; Gassner and Wilgus, 1940; Hughes, 1944; Goldsmith, Gordon and Charriper, 1945; Andrews and Schnetzler, 1945; Hollander and Riddle, 1946). Goiters in these studies resulted from iodine deficiency *per se*, thiourea, and thiouracil. In mammals the goiterogenic agents are transmitted to the young through the placenta and the milk (Hughes, 1944; Goldsmith, Gordon, and Charriper, 1945). In birds the agents are transmitted through the egg (Andrews and Schnetzler, 1945).

There is a lack of information on the effect of hyperthyroidism of the dam on the thyroid size of her progeny. In the present paper thyroid weights of newly hatched chicks from normal and thyroprotein-fed hens are compared.

METHODS

In January and February 1948, three hatches were made from two groups of 10-month old Rhode Island Red hens. One group of 80 hens had been fed 0.02% thyroprotein[1] in the ration continuously from day 1. A control group of 80 comparable hens had been fed the same basal ration. The previous history of these birds has been described in detail elsewhere (Wheeler, Hoffmann, and Graham, 1948; Wheeler and Hoffmann, 1948). The chicks were reared in electric brooders with raised screen floors and were fed a conventional all-mash starting ration. On day 2, 8, and 16 a sample of chicks was sacrificed, the thyroid glands removed and weighed to the nearest 0.1 mg. on a Roller-Smith balance.

RESULTS

The thyroid gland weights of the 2-day old male and female chicks from the three hatches are presented in Table 1. In every case the thyroid glands of the chicks from thyroprotein-fed hens are significantly heavier. A sex difference in response to the treatment is seen in the significantly greater thyroid enlargement in the female chicks. Figure 1 presents a composite curve of the change in thyroid weight from day 2 to day 16. Thyroid weight in the control chicks decreased somewhat during the first week and increased rather sharply during the second week after hatching. Thyroid weight in the experimental chicks decreased sharply during the first week and increased somewhat the second week. By the 16th day weights were comparable in both groups.

There were no observed differences in behavior, or in rate of bodily growth between the two groups of chicks.

TABLE 1. THYROID WEIGHTS OF 2-DAY OLD CHICKS FROM
THYROPROTEIN-FED HENS

Diet of hen[1]	No. of chicks	Sex	Av. thyroid wt. (mg.)	t value[2]	Date of hatch
Treated	5	M	9.60	5.93	
Control	5	M	4.96		
					1-1-48
Treated	5	F	10.08	4.09	
Control	5	F	4.68		
Treated	7	M	7.00	2.32	
Control	5	M	4.22		
					1-13-48
Treated	5	F	8.36	5.28	
Control	5	F	3.84		
Treated	5	M	6.30	3.56	
Control	9	M	4.00		
					2-5-48
Treated	5	F	8.70	4.03	
Control	5	F	3.90		
All treated	17	M	7.63	6.07	
All controls	19	M	4.39		
All treated	15	F	9.05[3]	7.35	
All controls	15	F	4.14		

[1] Diet of treated hens contained thyroprotein at 0.02% level.
[2] Calculated for the mean thyroid weight of each treated group and the corresponding control group.
[3] Thyroid weight of treated females significantly greater than that of treated males ($t = 2.1$).
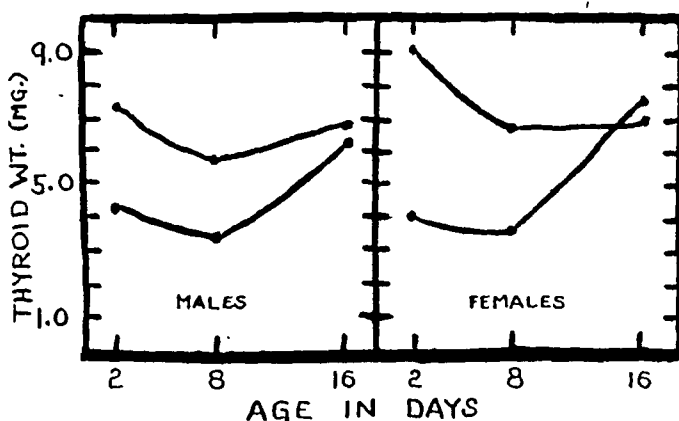
FIG. 1. Composite curve showing change in weight of thyroid from day 2 to day 16. The lower line in each case represents the control chicks, and the upper line the chicks from thyroprotein-fed hens.

COMMENT

The effect on the chick thyroid of feeding thyroprotein to the hen is the antithesis of the effect of feeding this substance directly to the chick or to the adult. Feeding thyroprotein directly results in marked thyroid involution (Wheeler, Hoffmann, and Graham, 1948; Wheeler and Hoffmann, 1948).

The results obtained here bear remarkable similarity to those occurring

when thiourea or thiouracil are administered to pregnant rats or to hens (Goldsmith, Gordon, and Charriper, 1945; Andrews and Schnetzler, 1945). With each of these treatments (thyroprotein, thiourea, thiouracil) the offspring have enlarged thyroids. Further similarity is seen in the return to normal thyroid weight when the chicks are fed a conventional ration. Goldsmith and coworkers reported that the enlarged thyroids of newborn rats from thiourea-fed mothers became reduced soon after the baby rats were fed a standard diet. The sex difference in response (greater thyroid enlargement in female chicks) reported here is similar to the observations of Glazener and Jull (1946) in thiouracil-fed chicks.

The goiterous chicks of thyroprotein-fed hens differ from the goiterous offspring of iodine-deficient dams. The latter progeny show marked debility and weakness (Kalkus, 1920; Wilgus and Gassner, 1941; Hollander and Riddle, 1946). The experimental chicks in our study were indistinguishable from normal chicks in vigor, behavior, and rate of growth.

We are unable to explain adequately the difference in post-hatching rate of growth of the normal chick thyroid shown in our data and the data of Albert, *et al.* (1947). The latter workers showed a regular increment in thyroid weight from day 1 to day 25, as contrasted to the initial decline at 8 days which we observed. Two factors might account for this difference: different breeds were studied, and the weights were taken at different seasons. Albert and coworkers obtained their data from White Leghorn males in May, June, and July, while our weights were from Rhode Island Red chicks in January and February.

These findings are admittedly preliminary and work is in progress to further study the mechanism involved in the production of goiterous chicks from hyperthyroid dams.

SUMMARY

Chicks from hens fed 0.02% thyroprotein (containing 3.0% thyroxin by weight) have significantly enlarged thyroids at the time of hatch. When these chicks are fed a standard ration their thyroids become reduced within the normal range by the 16th day following hatch. These results are of interest since thyroprotein fed directly to chicks or to adults produces marked involution of the thyroid.

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NEW BOOK

GEORGE CRILE, An Autobiography. Edited, with Sidelights, by Grace Crile. In Two Volumes. J. B. Lippincott Company, Philadelphia and New York. 1947.

This is an extraordinary autobiography. From humble origins, with only those educational opportunities which he could earn for himself, George Washington Crile rose to the foremost rank of American and international surgery; he served his country well in two wars, being one of the few medical reserve officers ever honored with the grade of Brigadier General; and together with his lifelong friend, "Ed" Lower, he founded one of the great surgical clinics of the world. These accomplishments were not in themselves primary objectives. They came as the logical result of Dr. Crile's prodigious industry. His primary interest in life was to learn the how and why of things. His mind was keen, tireless, fearless, and passionately inquisitive. When he could not see or find the answer to a problem he dreamed up an answer.

No biographer could have portrayed the nature and direction of Dr. Crile's thinking as well as this collection of personal notes and sketches, scribbled hastily on bits of paper whenever the author had a moment on a train, a street car, or even in the bath tub. The material has been assembled and edited by Mrs. George Crile who has contrived to give a cohesiveness to the story without destroying the basic character of the original notes.

These are not the profound reflections of a great man looking back over a life of memorable accomplishment. These are the thoughts, the hopes, and the works of a man, forever young, constantly looking ahead, driving, seeking, finding, dreaming, and living. The incidents are succinctly but often minutely described and their range is stupendous: a hair raising description of intubating a child with diphtheria in the days when Dr. Crile was a "horse and buggy doctor"; the first total laryngectomy done in America; early experiences in spinal anesthesia; studies in shock and in thyroid disease; experiences in two wars; hunting big game in an extraordinary study of comparative anatomy and physiology; and through it all a constantly recurring glimpse of deep, happy family associations.

There is struggle, disappointment, and, as in the great Cleveland Clinic fire, disaster, but there is little sadness and no bitterness in these pages. The loneliness and frustration which so obviously gripped Harvey Cushing in his last years were completely unknown to Crile. He never accepted defeat. He overcame it or rationalized it out of his ken. He mastered his impending blindness by developing an operation for hypertension which could be done by touch alone. At 75, practically blind, harassed by personal financial difficulties, his son, Barney, departing for war, and his Clinic facing possible disintegration, he had the amazing intellectual vigor to take an active and instrumental part in developing a pressurized flying suit for the navy.

The editorial sidelights by Mrs. Crile contain some of the best writing in the book and give an insight to Dr. Crile's life which would be missed without them.

II. 2:00 P.M. Red Lacquer Room

C. N. H. LONG, *presiding*

12. A SIMPLIFIED HYPOPHYSECTOMIZED RAT ADRENAL ASCORBIC ACID BIO-
ASSAY METHOD FOR ADRENOCORTICOTROPHIN (A.C.T.H.); SPECIFICITY
AND APPLICATION TO PREPARATIVE PROBLEMS.
   by Paul L. Munson, Alfred G. Barry, Jr. (by invitation), and F. C.
   Koch

13. CONTENT OF ADRENOCORTICOTROPHIC HORMONE (A.C.T.H.) IN THE RAT
PITUITARY UNDER OPTIMAL AND STRESSFUL ENVIRONMENTAL CONDI-
TIONS.
   by George Sayers, Marshal Merkin (by invitation) and J. N. Tortoreto
   (by invitation)

14. THE ACTIVATION OF THE ADRENAL CORTEX BY INSULIN HYPOGLYCEMIA.
   by H. Gershberg (by invitation) and C. N. H. Long

15. INFLUENCE OF ADRENOTROPHIC HORMONE ON SODIUM EXCRETION IN
HYPOPHYSECTOMIZED RATS.
   by Betty L. Rubin (by invitation) and Ralph I. Dorfman

16. FACTORS INFLUENCING THE CORTICOTROPHIN PRODUCTION OF THE AN-
TERIOR PITUITARY.
   by Hans Selye

17. THE USE OF ADRENOCORTICOTROPHIN AS A TEST OF ADRENAL CORTICAL
RESERVE.
   by George W. Thorn, Peter H. Forsham (by invitation), Lillian Recant
   (by invitation) and A. Gorman Hills (by invitation)

18. OBSERVATIONS ON THE PITUITARY-ADRENAL RESPONSE FOLLOWING
EPINEPHRINE INFUSION IN MAN.
   by Lillian Recant (by invitation), Peter H. Forsham (by invitation)
   and George W. Thorn

19. FATE AND METABOLIC ACTION OF INTRAVENOUSLY ADMINISTERED
ADRENOCORTICOTROPHIC HORMONE (A.C.T.H.).
   by Thomas W. Burns (by invitation), George Sayers, Frank H. Tyler
   (by invitation), B. V. Jager (by invitation), T. B. Schwartz (by invita-
   tion), Emil L. Smith (by invitation) and L. T. Samuels

20. METABOLIC CHANGES FOLLOWING THE ADMINISTRATION OF PITUITARY
ADRENOCORTICOTROPHIC HORMONE (A.C.T.H.) TO NORMAL HUMANS.
   by H. T. McAlpine (by invitation), E. H. Venning, L. Johnson (by in-
   vitation), V. Schenker (by invitation), M. M. Hoffman and J. S. L.
   Browne

21. THE EFFECT OF ADRENOCORTICOTROPHIN ON ANTIBODY LEVELS IN NOR-
MAL HUMAN SUBJECTS.
   by P. H. Herbert and J. A. de Vries (introduced by J. S. L. Browne)

22. A COMPARISON OF THE EFFECT ON BONE FORMATION OF THE HYPER-
ADRENOCORTICISM OF CUSHING'S SYNDROME WITH THAT INDUCED BY
ADRENOCORTICOTROPHIC HORMONE (A.C.T.H.).
   by Frederic C. Bartter (by invitation), Anne P. Forbes and Fuller Al-
   bright

23. ADRENAL CORTICAL UNRESPONSIVENESS IN PATIENTS WITH GASTRIC
CANCER.
   by Edward C. Reifenstein, Jr., N. F. Young (by invitation), Aurelia
   Potor (by invitation), Benedict Duffy (by invitation) and F. Hom-
   burger (by invitation)

24. THE EXCRETION OF ADRENAL METABOLITES IN HUMAN URINE.
   by Konrad Dobriner, Seymour Lieberman (by invitation) and C. P.
   Rhoads (by invitation)

III. Annual Dinner

    7:30 p.m.—Red Lacquer Room, Palmer House
        Presentation of E. R. Squibb and Sons Award for 1948.
        Presentation of Ciba Award for 1948.
        Presentation of Ayerst, McKenna and Harrison Fellowship for 1948.
        Warren O. Nelson, Chairman of the Committee on Awards 1947–48.

        President's Address: C. N. H. Long, Yale University

SATURDAY, JUNE 19, 1948

IV. 9:00 a.m. Red Lacquer Room

    R. G. Hoskins, *presiding*

    25. PREPARATION OF CRYSTALLINE GROWTH HORMONE.
        by Jacob B. Fishman (by invitation), Alfred E. Wilhelmi (by invitation) and Jane A. Russell
    26. THE INFLUENCE OF PURIFIED GROWTH HORMONE ON FASTING METABOLISM.
        by Clara M. Szego and Abraham White
    27. UNPREDICTABLE EFFECTS OF GROWTH HORMONE PREPARATIONS ON NITROGEN STORAGE.
        by Paul Bartlett (by invitation) and Oliver H. Gaebler
    28. STUDIES IN GROWTH. I. THE EFFECTS OF ANDROGEN IN GIGANTISM AND ACROMEGALY.
        by Lawrence W. Kinsell, George D. Michaels (by invitation), Choh Hao Li (by invitation) and William E. Larsen (by invitation)
    29. THE EFFECT OF IODINE INJECTIONS ON ENERGY METABOLISM AND PLASMA PROTEIN-BOUND IODINE OF RATS.
        by S. B. Barker and H. J. Lipner (by invitation)
    30. THE EFFECT OF PITUITARY AND NON-PITUITARY GLAND FACTORS ON THE FORMATION OF INTRACELLULAR COLLOID DROPLETS IN THE THYROID GLAND IN VIVO AND IN VITRO.
        by Samuel Dvoskin
    31. INACTIVATION OF THE EXOPHTHALMIC, THYROTROPIC AND KETOGENIC PRINCIPLES OF ANTERIOR PITUITARY EXTRACT BY IODINATION.
        by William McK. Jefferies
    32. NEWER METHODS OF ANTAGONIZING HYPERTHYROIDISM.
        by Robert H. Williams, Rene F. Tagnon (by invitation), Herbert Jaffe (by invitation), Beverly T. Towery (by invitation) and Walter F. Rogers (by invitation)
    33. THE USE OF RADIOACTIVE IODINE (I131) IN THE STUDY OF NORMAL AND DISORDERED THYROID FUNCTION IN MAN.
        by Sidney C. Werner and Edith Quimby (by invitation)
    34. THE EFFECT OF THYROID STIMULATING HORMONE ON THE FUNCTION OF HUMAN NORMAL AND MALIGNANT THYROID TISSUE.
        by J. B. Trunnell (by invitation), R. W. Rawson, L. D. Marinelli (by invitation) and Ruth Hill (by invitation)
    35. THE RELATION BETWEEN INFANT BIRTHWEIGHT AND SUBSEQUENT DEVELOPMENT OF MATERNAL DIABETES MELLITUS.
        by Joseph P. Kriss and Palmer H. Futcher (introduced by Cyril M. MacBryde)

V. 2:00 p.m. Red Lacquer Room

    A. T. Kenyon, *presiding*

    36. ABSORPTION AND EXCRETION OF CHORIONIC GONADOTROPHIN WHEN ADMINISTERED INTRAMUSCULARLY TO WOMEN.
        by J. T. Bradbury and Willis E. Brown

37. The Renal Clearance of Chorionic Gonadotrophic Hormone in Pregnancy and in Neoplasm of the Testis.
    by C. F. Gastineau (by invitation), A. Albert and L. M. Randall (by invitation)
38. The Metabolic Response to Chorionic Gonadotrophin in Young Men.
    by Kathryn Knowlton (by invitation) and Allan T. Kenyon
39. Blood Gonadotrophin Studies During Pregnancy in Relation to the Fetal Sex.
    by H. E. Niegurgs and Robert B. Greenblatt
40. On the Principal Estrogenic Constituents of the Urine of the Stallion.
    by Louis Levin
41. Mechanism of Inactivation of α-Estradiol by Rat Liver in Vitro.
    by R. H. deMeio (by invitation), A. E. Rakoff, A. Cantarow and K. E. Paschkis
42. Cozymase in the Hepatic Inactivation of α-Estradiol.
    by Richard L. Coppedge (by invitation), Albert Segaloff, Herbert Sarett (by invitation) and Aaron Altshul (by invitation)
43. Interference with Estrogen-Induced Growth in the Female Genital Tract by Folic Acid.
    by Roy Hertz
44. The Relation of Folic Acid to the Action of Estrogens.
    by Irene T. Kline (by invitation) and Ralph I. Dorfman
45. Fluorescent Phenomena of the Vulva Associated with Sex Hormone Metabolism.
    by M. Sydney Margolese
46. Testicular Deficiency: A Clinical and Pathological Study.
    by R. Palmer Howard, Ronald C. Sniffen (by invitation) and Fred A. Simmons
47. A Comparison of the Effect of Various Androgens on the Temporal Muscle and Organs of the Castrated Male Guinea Pig.
    by Charles D. Kochakian and Jane Harrison Humm (by invitation)

VI. Annual Business Meeting
5:00 p.m. Red Lacquer Room

Papers Read by Title

48. The Use of Whole Adrenal Cortical Extract in Experimental Infections.
    by Erwin P. Vollmer, James D. Gillmore (by invitation), Leo Cravitz (by invitation) and J. E. Samsell (by invitation).
49. The Work Performance of Adrenalectomized Rats Given Continuous Intravenous Infusions of Glucose.
    by Dwight J. Ingle and James E. Nezamis (by invitation)
50. Sublinqual Administration of Desoxycorticosterone Acetate in the Treatment of Addison's Disease.
    by Evelyn Anderson, Lawrence W. Kinsell, Troy C. Daniels (by invitation) and Edward Henderson .
51. Excretion of Adrenal Metabolites Following the Administration of Adrenocorticotrophic Hormone to Normal Human Subjects.
    by Eleanor H. Venning, V. E. Kazmin (by invitation), Miriam Ripstein (by invitation), H. T. McAlpine (by invitation) and M. M. Hoffman
52. The Effect of 11-Dehydrocorticosterone on Fecal Fat Excretion.
    by Grace E. Bergner (by invitation), Roger A. Lewis (by invitation),

Frances W. Stout (by invitation), George W. Thorn and Kendall Emerson, Jr.

53. AN ESTIMATION OF THE QUANTITY OF 11-17-OXYSTEROID EXCRETION BY THE HUMAN ADRENAL STIMULATED BY A.C.T.H.

by A. Gorman Hills (by invitation) and George W. Thorn

54. ISOLATION OF URINARY STEROIDS FROM A PATIENT WITH APPARENT ADRENAL INVOLVEMENT.

by A. M. Miller (by invitation) and Ralph I. Dorfman

55. THE EFFECT OF ADRENALECTOMY AND DESOXYCORTICOSTERONE ACETATE ADMINISTRATION UPON THE ECG RESPONSE OF THE RAT TO CARDIAC GLYCOSIDES.

by Herbert S. Kupperman, Joseph G. Benton (by invitation) and Arthur C. DeGraff (by invitation)

56. ACTIVATION OF THE ADRENAL CORTEX IN HUMAN SUBJECTS FOLLOWING ELECTROCONVULSIVE THERAPY (E.C.T.) AND PSYCHOMOTOR STRESS.

by R. A. Cleghorn and A. J. Goodman (by invitation), B. F. Graham, M. H. Jones and N. K. Rublee

57. EFFECT OF ADRENAL CORTICAL COMPOUNDS ON ELECTROLYTE METABO-LISM OF A PATIENT WITH ADDISON'S DISEASE DURING HIGH SODIUM CHLORIDE INTAKE.

by Aurelia Potor (by invitation), Nelson F. Young (by invitation), F. Homburger (by invitation) and Edward C. Reifenstein, Jr.

58. NITROGENSPARING (PROTEIN-ANABOLIC) ACTION OF THYROID HORMONE.

by J. Rupp (by invitation) and K. E. Paschkis

59. THIOURACIL EFFECT ON PLASMA AND LIVER PROTEIN CONCENTRATIONS.

by James H. Leathem

60. RADIOIODINE UPTAKE BY THE THYROID AS AN AID IN DIFFERENTIAL DIAGNOSIS.

by S. M. Seidlin, E. Oshry (by invitation), I. Rossman (by invitation) and L. Leiter (by invitation)

61. THYROID UPTAKE OF RADIOACTIVE IODINE IN THE NORMAL AND HYPO-METABOLIC HUMAN.

by Martin Perlmutter (by invitation) and Peter H. Forsham (by invitation)

62. CELLULAR INVOLUTION IN THE THYROID.

by Nathan B. Friedman

63. METHYL THIOURACIL IN THE TREATMENT OF THYROTOXICOSIS.

by Grosvenor W. Bissell, John M. Benny (by invitation), Victor Totah (by invitation) and Florence Gilbert (by invitation)

64. MODIFICATION OF THE ESTRUAL CYCLE OF THE EWE BY THE USE OF PROGESTERONE; THE EFFECT UPON SUBSEQUENT OVULATION RATE AND FERTILITY OF OVA.

by R. H. Dutt (by invitation) and L. E. Casida

65. EFFECTS OF VARIOUS ESTROGENIC PREPARATIONS ON THE VAGINAL MUCOSA.

by Mildred Vogel (by invitation), Thomas H. McGavack and Joseph Mellow (by invitation)

66. THE USE OF THE VAGINAL SMEAR IN THE ASSAY OF ESTROGENS GIVEN ORALLY OR INTRAMUSCULARLY.

by Willis E. Brown and J. T. Bradbury

67. THE SIMILARITY OF ESTROGENIC EFFECT IN PREMENSTRUAL TENSION, MENSTRUAL ANOMALIES, CHRONIC CYSTIC MASTITIS AND CANCER OF THE BREAST.

by Joseph H. Morton

68. HYPERESTROGENISM TREATED WITH LACTOGENIC HORMONE (PROLACTIN).

by Manuel Villaverde

69. The Factor of Rhythm in Experimental Menstruation.
    by Doris H. Phelps
70. Hormonal Pellets in the Management of the Menopausal Syndrome.
    by Robert B. Greenblatt and Roland R. Suran (by invitation)
71. The Effect of Hypophysectomy on the Ovulability of the Ovarian Follicle of the Domestic Hen.
    by Irving Rothchild and R. M. Fraps
72. Prognostic Value of Pregnandiol Excretion in Threatened Abortion with Special Reference to the Effects of Diethstilbestrol.
    by A. R. Abarbanel
73. Further Studies on the Endometrial Cups of the Pregnant Mare.
    by H. H. Cole and G. H. Hart
74. Inactivation of Posterior Pituitary Antidiuretic Hormone of the Liver.
    by W. J. Eversole, J. H. Birnie (by invitation) and Robert Gaunt
75. The Estimation of Dehydroisoandrosterone and Related Compounds in Human Urine by a Modification of the Pettenkoffer Reaction.
    by Richard L. Landau and Kathleen Lugibihl (by invitation)
76. Clinical Evaluation for 17 Ketosteroids by the Rapid Method.
    by T. H. McGavack, S. Kenigsberg, A. M. Shearman and K. J. Drekter
77. The Application of Paper Partition Chromatography to Ketosteroids.
    by Robert B. Burton (by invitation), Alejandro Zaffaroni (by invitation) and E. Henry Keutmann
78. A Rapid Modification of the Zimmerman Test for Ketosteroids.
    by Sidney Pearson and Sylvester Giaccone (by invitation)
79. A Fluorometric Method for the Determination of Estrone and Estradiol in Human Urines.
    by Joseph W. Jailer
80. A Clinical Bio-Assay for Chorionic Gonadotrophin.
    by A. Albert
81. Specificity of a Colorimetric Method for Dehydroisoandrosterone in Urine Extracts.
    by Paul L. Munson, Mary Ellen Jones (by invitation), Philip J. McCall (by invitation) and T. F. Gallagher (by invitation)
82. The Colorimetric Determination of Sodium and Its Application in the Study of Sodium and Chloride Balance.
    by Joseph W. Goldzieher and Gilbert Stone (by invitation).
83. Depression of Lymphocyte Count after Orally Administered Glucose.
    by Paul A. Marks (by invitation), Dorothy T. Marks (by invitation) and Joseph W. Jailer
84. Pitfalls in the Diagnosis of Diabetes.
    by Bernard A. Watson
85. Electrolyte Balance Studies in the Uncontrolled and Controlled Diabetic State.
    by Jonas Weissberg (by invitation), Thomas H. McGavack, A. M. Shearman (by invitation) and I. J. Drekter
86. The Role of the Endocrine Glands in Body Temperature Regulation.
    by H. E. Nieburgs and Robert B. Greenblatt
87. The Lipogenic Activity of Propythiouracil, Triphenylchloroethylene and Hexestrol in Chickens.
    by R. George Jaap

88. THE INFLUENCE OF THE PITUITARY GLAND ON BLOOD VESSEL DEVELOPMENT. THE EFFECT OF CONCENTRATED PITUITARY EXTRACT ON THE ISOLATED KIDNEY.

by Robert C. Moehlig and Louis Jaffe (by invitation)

89. RESULTS OF PROLONGED MEDICAL TREATMENT OF OBESITY WITH DIET ALONE, DIET AND THYROID PREPARATIONS, AND DIET AND AMPHETAMINE.

by David Adlersberg and Martin E. Mayer (by invitation)

90. THE URETHRAL SMEAR IN THE NORMAL HUMAN MALE.

by Mildred T. Vogel (by invitation), Thomas H. McGavack and ·Henry Kammandel (by invitation)

91. THE SPECIFIC OVARIAN HYPEREMIC INDUCING EFFECT OF LUTEINIZING AND LUTEOTROPHIC HORMONES.

by Herbert S. Kupperman, W. H. McShan and Roland K. Meyer

GRAFTED MOUSE OVARIES AND THEIR ADRENAL CORTICAL FUNCTION[1]

R. T. HILL

From the Department of Anatomy, Indiana University School of Medicine

BLOOMINGTON, INDIANA

INTRODUCTION

ALTHOUGH it has long been suspected that the ovary is responsible for some degree of androgenic activity, Lipschutz (1932) was apparently the first to present good evidence of such activity. A female guinea pig bearing a grafted ovary took on several characteristics of maleness, including a greatly overdeveloped clitoris. Lipschutz ascribed this unusual development to the presence of the grafted ovary. However, this isolated case did not constitute proof of androgenic activity by the ovary. More recent experiments with mice (Hill and Gardner, 1936; Hill, 1937; Hill and Strong, 1938; 1940) greatly extended the studies on the androgenic activity of ovaries, and have given final proof to the original idea of Lipschutz. The environmental temperature of the graft was shown to be at least one factor in the control of the androgenic activity of ovaries, but the specific androgen produced has never been discovered. Deanesly (1938) found that some androgenic activity occurred when ovaries were grafted to the ears of rats. However, Deanesly believed that thecal luteinization rather than temperature was the critical factor. Although the bulk of experimental work has been done with mice, sufficient evidence has been obtained from other animals to indicate that the reaction is not species specific.

In our experiments with mice it has been noted that the histological appearance of grafted and androgenically active ovaries is not normal. Cords of cells are often noted which suggest a rete arrangement, but these particular cords of cells can be traced and are found to be infoldings from the germinal epithelium. Also it has been noted

that a majority of grafted ovaries contain clusters of "lutein like" cells, though these clusters are not normal lutein cells, nor are they a part of normal corpora lutea. Furthermore the majority of grafted ovaries have cells present which cannot be classified as one of the cell types normally found in mouse ovaries. In fact these unusual cells appear more like some that are frequently found in the medulla and cortex of the adrenal than in any other tissue of the mouse. Ordinary staining methods do not differentiate these adrenal-like cells. It is not thought that these unusual cells are embryonic rests of cortical cells because they are not found in normal mouse ovaries. It was the striking similarity of these two different groups of cells, coupled with the extremely close proximity of origin of the gonad and adrenal cortex in the embryo, that gave rise to the experiments reported here and in an earlier preliminary paper (Hill, 1944). In the preliminary note the number of animals used was small. The present data are substantial in numbers; they corroborate our earlier findings, and further extend the problem.

EXPERIMENTAL

A total of 279 animals were used in the experiments reported in this paper. They comprised 105 control animals and 174 experimental animals. The mice were of two strains: Swiss Albinos and the CHI strain obtained from the inbred colony of Dr. L. C. Strong. The Swiss Albino mice were used for the purpose of testing whether the reactions obtained were strain specific. In these experiments ovaries were grafted into the ears of male mice or into one testis of male mice, or were autografted into the ears of female mice. Subsequent to grafting the ovaries an interval of two or more weeks was allowed before the removal of adrenal tissue. All adrenalectomies were done in a two stage operation, the right adrenal being removed first in each instance. The left adrenal was removed from one to two weeks following ablation of the right gland. Experimental and control animals were of the same general age, each animal being between 100 and 200 days old at the time of adrenal removal. The ovary graftings were done when all animals were between 75 and 100 days of age.

Control animals: The control group was comprised of 105 animals, 38 of which were CHI females, 37 were CHI males, 11 were Swiss Albino females, and 19 Swiss Albino spayed females. All control animals, completely adrenalectomized, died within 29 days following removal of the second adrenal. The peak of mortality came on the 7th, 8th, and 9th days post adrenalectomy (Fig. 1). The average survival time of the CHI females was 11.5 days and of the CHI males was 11.4 days—essentially identical intervals. The post adrenalectomy survival period of the albino females was 12.0 days, an interval not significantly greater than that survived by animals of the CHI strain. The post adrenalectomy survival of the 19 spayed albino females was 6.7 days, a period quite definitely shorter than for any of the

other groups of control animals. Thus it is evident that there is no sex difference in survival periods of males and females of the CHI strain. Likewise there is no significant difference in survival periods of the two strains used in these experiments when the gonads are intact. The apparent shorter survival interval of the spayed albino females may be attributable to the absence of the gonads. However, the number of animals in this group was so small and the variation of survival was so great that we are not inclined to place any significance in the shorter survival period.

The weights of the normal control animals drop precipitously subsequent to adrenalectomy, followed by death of the animal. The
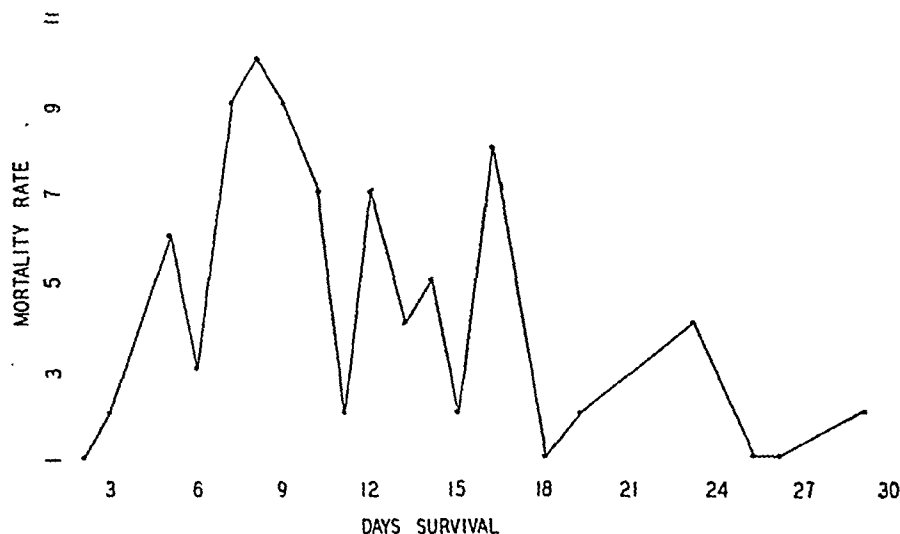
Fig. 1

weight decline of a typical control animal is of the same nature as that of experimental animal MSV 389 (Fig. 2). The loss of weight, and the downward incline of the weight curve is directly related to the survival period of the animal following adrenal removal. Sodium chloride added to the drinking water in concentrations of 0.5% and 1.0% did not affect the survival period. Two albino spayed females were killed about 45 days after removing the second adrenal gland, and found to be incompletely adrenalectomized. They are not included in our data on survival. Therefore 105 of 107 control animals proved to be completely adrenalectomized. We are here assuming that any mouse which survived less than 29 days after removal of the second adrenal gland has been completely adrenalectomized, and our data substantiates that assumption. Thus the completeness of adrenalectomies in these control experiments stands at slightly more than 98%.

Experimental animals: Group 1. The group of females bearing autografts of ovaries in their ears was made up of 89 CHI and 19

Swiss Albino animals. These animals may be divided into smaller groups according to survival periods following adrenalectomy. Fifty-four animals died during the first 29 days following adrenal removal, thereby not living beyond the maximum survival interval of the control animals. Thirty-five animals survived adrenalectomy beyond the 29 day control interval, but died at a somewhat later period without having undergone any further experimentation. Those animals which

MSV·389
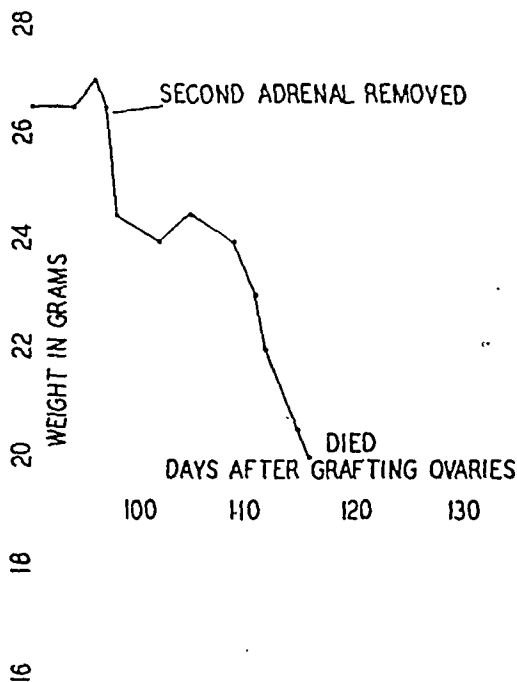
FIG. 2

died after extended survival lived an average of 121 days post adrenalectomy, the longest interval being 284 days. Two of the remaining animals were killed accidentally. Sixteen animals had grafts removed and survived an average of 38 days following graft removal. One animal (MSV) 508 which did not have its grafts of ovaries removed lived for 435 days following adrenalectomy. At death a large mammary tumor was present, and no adrenal cortical tissue could be found.

Group II. A total of 38 animals made up this group. All were CHI males which had two ovaries grafted into one (right) testis. Sixteen individuals, following removal of the suprarenal glands, died within the control survival period of 29 days. The six animals which died following extended survival lived an average of 94 days, the greatest

period for an individual being 199 days. Eleven animals had their grafts of ovaries removed by excision of the right testis, and survived an average of 44 days. Three 'animals were still alive and in good health at 180 days following removal of grafted ovaries, and were then killed for post mortem study. All three of these animals were found to have relatively large amounts of cortical tissue remaining. The

MSV 399
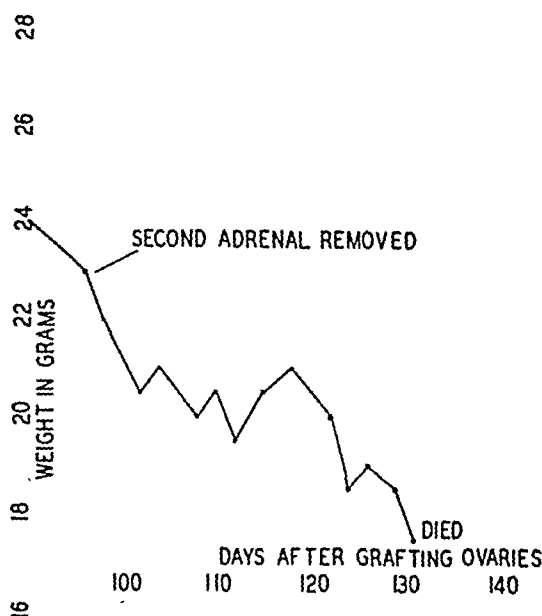
FIG. 3

remnants of cortical tissue were obviously responsible for the greatly extended survival in these three instances.

One animal, MSV 549, had its grafted tissue removed 330 days after adrenalectomy, and died on the 15th day following graft removal. Adrenal removal was complete. This animal was approximately 465 days old at death.

Of the four remaining animals, one was killed accidentally, and three, MSV 529, 545, 547, died with intact grafts of ovaries, at 262 days, 210 days, and 291 days, respectively, after removal of the second adrenal gland. In each of these three cases adrenal removal had been complete.

Group III. This group, the smallest of the three, included 28 CHI strain animals, all being males bearing grafts of ovaries in their ears. Subsequent adrenal removal, 17 animals died within the control period

of 29 days and 3 animals survived an average period of 56 days. Eight animals had their grafts removed, four dying during an interval ranging from 36 to 109 days later. Each of these animals died at an inopportune time, and so were not examined for remaining cortical tissue. The four animals that did not die were killed at 180 days post adrenalectomy, and cortical tissue was found to be present in each animal.

During the greater portion of the time most of the animals on experiment were weighed once or twice weekly. Some animals were weighed daily during critical periods. The curves obtained by plotting the weights are indicative of the general condition of the animal. Several typical curves of weights are shown here, and seem worthy of further consideration.
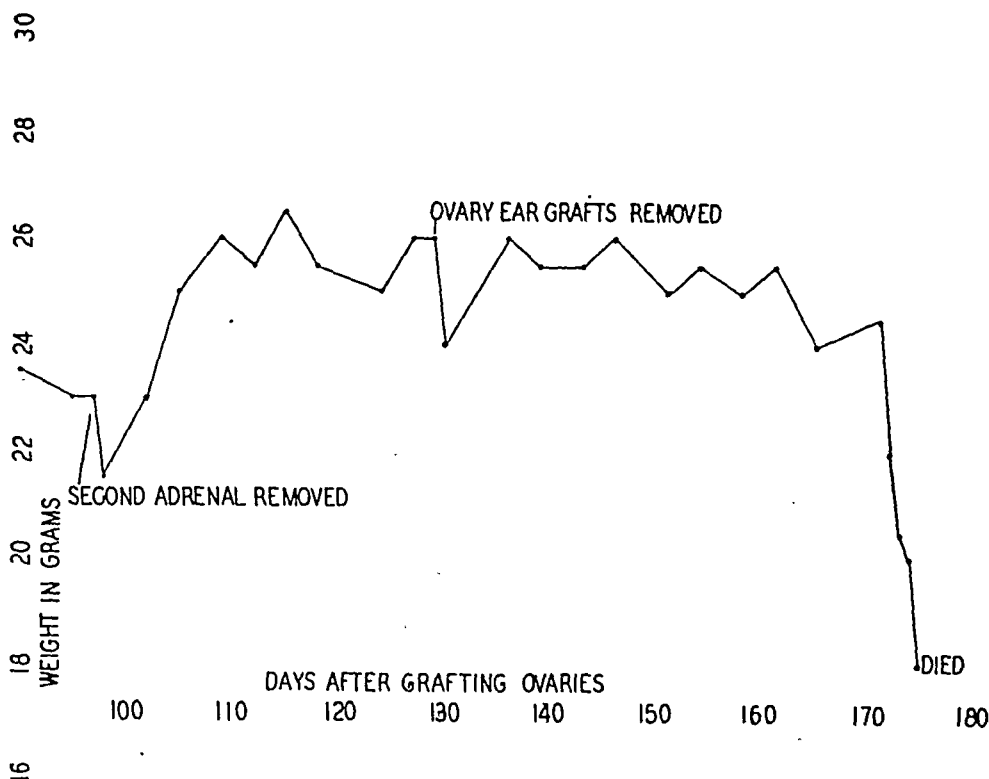
FIG. 4

Figure 2 shows weight changes of a spayed female (MSV 389) bearing autografts of ovaries in her ears. About 100 days after making the grafts, the adrenal glands were removed in a two-stage operation. The subsequent weight decline was very precipitous, followed by death 20 days after complete adrenalectomy. The curve obtained from this animal's weight changes is essentially identical with that obtained from any control animal. In this case the grafted ovaries were of no protection against the loss of the animal's own adrenal tissue. The

weight changes of animal MSV 399 (Fig. 3), likewise a spayed female
bearing ear autografts of ovaries, is very similar to the preceding one,
showing only a very slight and insignificant increase in survival time.
Animals MSV 392 and 400 (Figs. 4 and 5) were both spayed females
bearing grafts of their own ovaries in their ears. The weight changes
of both animals are very similar. Both animals showed a steady and
definite weight increase subsequent to adrenal removal until the time
of removal of the grafted ovaries thirty-two days later. The interval
of thirty-two days was used in these animals because during a slightly
lesser period (29 days) all of the control animals had lost a great deal
of weight and had died. Subsequent to removal of the grafted ovaries
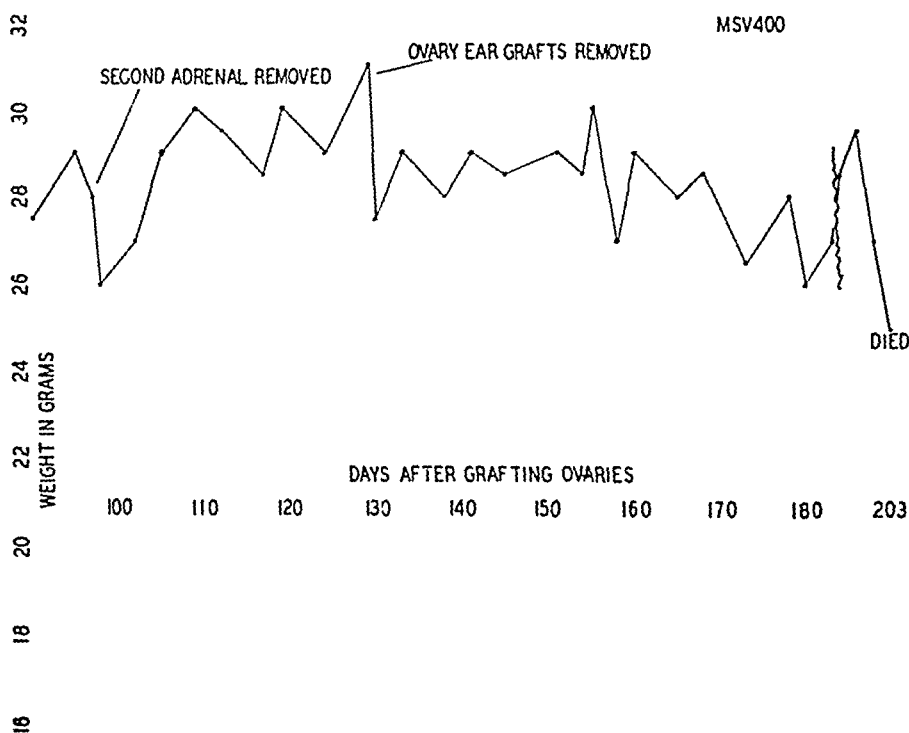
Fig. 5

both animals showed a definite downward trend in weight and died
at intervals of 49 and 74 days later. Both of these animals, and their
weight changes, indicate that the grafted ovaries provided a protec-
tion of some kind against the loss of the host animal's adrenal tissue.
An obvious question arises as to the extended survival period follow-
ing removal of the grafted ovaries. We believe it may be assumed that
both of these animals carried some amount of accessory cortical tissue,
and that the accessory cortical tissue was given time to undergo hy-
pertrophy while death soon after adrenal removal was at least de-
layed by the presence of the grafted ovaries. Our belief in the presence
of accessory cortical tissue and its ability to prolong survival follow-

cortical function for a long period may not be histologically distinguishable from a grafted ovary which did not prolong life at all in its adrenalectomized host animal. Figure 9 is a photomicrograph of a section of the grafted ovary taken from MSV 517, an animal which survived adrenalectomy 60 days, dying 3 days after graft removal. Figure 10 shows a section from the grafted ovary of MSV 397. This mouse survived 8 days after adrenalectomy. No distinctly different characters can be found in these two grafts. New stains and new staining techniques are being tried in an effort to discover some possible correlation between structure and function.

MSV 494
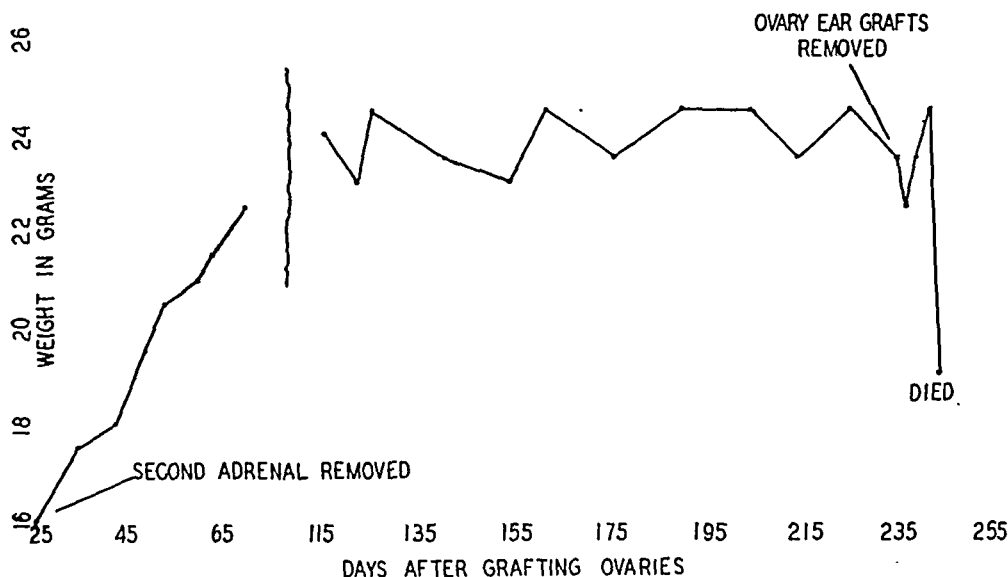
FIG. 8

Perhaps it is not surprising that no correlation has been forthcoming. In the extensive experiments which show androgenic activity of grafted ovaries no correlation has been found between the structure of the graft and its androgen output. The grafts of ovaries which produce such a marked life sustaining adreno-cortical function are quite identical with those grafts which have replaced the androgenic function of testes. It was found that all ovaries grafted to the ear, which had become well established, were capable of producing androgens (Hill, 1937). In our studies of the adrenal cortical life-sustaining function of grafted ovaries the percentage of positive responses is markedly less than 100%. At present we can offer no explanation as to why the reaction is successful in maintaining the life of only a part of our animals. In some of our earlier experiments some animals were kept at either 27°C. or 21°C. with no obvious effect on survival following

adrenalectomy. These data, with information from additional experiments, have proven that temperature is not a factor in causing grafted ovaries to exhibit an adrenal cortical function.

It is not possible to obtain fresh tissue from every animal at death. An animal may look very lively in the late afternoon, but be dead and unusable for histological study by morning. Thus tissues were not collected from every animal.

Control animals with apparent complete lack of remaining cortical tissue usually do not survive more than 10 to 14 days; those with very small clusters of cortical cells remaining will survive slightly

Fig. 9. Photomicrograph of grafted ovary removed from MSV 517. ×100.

longer, up to 20 or 30 days following adrenalectomy. When relatively large amounts of cortex are found the survival has been more extended ranging from around 40 days upward to more than 100 days. Two female mice bearing autografts of ovaries in their ears, each survived graft removal 44 days, and each animal had moderate-sized remnants of cortical tissue at death. Our evidence leads us to believe that extended survival time in control adrenalectomized mice bears a direct correlation with the mass of remaining cortical tissue. The cortical tissue found at post mortem has either been left behind during sur-

gery, or has formed from small embryonic rests of cortical cells following removal of the main suprarenal gland.

The three of the more striking instances in this group of experiments are those of animals MSV 494, MSV 508, and MSV 549. Animal MSV 494 (Fig. 8) lived 213 days following complete adrenalectomy, and then died 7 days following removal of grafted ovaries. One mouse (MSV 508) lived 435 days subsequent to complete adrenalectomy, having intact grafted ovaries during the entire period. MSV 549 lived 330 days after complete adrenalectomy, and died on the 15th day

FIG. 10. Photomicrograph of grafted ovary removed from MSV 397. ×100.

after removal of the grafted ovaries. To these three animals should be added the many others which survived beyond the survival period of control adrenalectomized mice, and yet died soon after removal of grafted ovaries. In the three individual cases listed above there remains no doubt but that the extended survival beyond complete adrenalectomy was a response to the life-sustaining action of grafted ovaries.

A total of 174 mice were used in the experiments recorded here (exclusive of control mice). Of this total number, one half (87) died within the first days 29 following adrenal removal, and therefore were not benefited significantly by the presence of grafted ovaries. Thirteen

of the remaining half were found to have adrenal cortical tissue rem-
nants at post mortem, and can therefore be excluded from any final
tabulation. Seventy-four mice lived longer than the 29 day maximum
survival period of the controls. Not all of these 74 animals could be
examined for the presence of adrenal cortical tissue, but the majority
were found to be completely adrenalectomized. Thus about 45% of
our experimental animals, living longer than the 29 day control inter-
val, were materially benefited by the presence of grafted ovaries and
their ability to produce a life-sustaining adreno-cortical function.
Fifty-five percent of our animals received no significant help from the
presence of grafted ovaries. Ninety-eight per cent of our control mice
were so completely adrenalectomized as to cause their death within
29 days following removal of the second adrenal gland.

SUMMARY

Data are presented which show that under the conditions of these
experiments mouse ovaries (grafted) can maintain life, in a host
mouse, in the complete absence of adrenal cortical tissue. Normal
mice, both males and females, do not survive beyond four weeks in
the complete absence of adrenal cortical tissue. Intact gonads and
gonad removal in mice does not significantly alter survival time. Re-
moval of the grafted ovaries from completely adrenalectomized mice
results in death. No histological or chemical explanation is presented.

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CHOLESTEROL STUDIES OF ADRENALS AND PLASMA IN PARTIALLY HEPATECTOMIZED AND PARTIALLY NEPHRECTOMIZED RATS AFTER INJECTION OF HN3[1]

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THE FACTORS involved in the regulation of cholesterol metabolism are poorly understood. In previous papers (Ludewig and Chanutin, 1946), (Ludewig and Chanutin, 1947), (Chanutin and Ludewig, 1947) it was shown that intravenous injection of tris (β-chloroethyl) amine (HN3) into intact rats causes a marked and prolonged decrease in the ester cholesterol content of the adrenals and only slight changes in the plasma cholesterol concentration. It is known that the concentration and the distribution of plasma cholesterol is altered in cases of liver or kidney insufficiency in rats (Chanutin and Ludewig, 1936), (Ludewig and Chanutin, 1938). In the present investigation, the cholesterol metabolism was studied after imposing a specific injury by means of HN3 in animals with experimentally produced liver or kidney insufficiency.

METHODS

Inbred male albino rats, 60–70 days of age, of Wistar strain, were used as experimental animals. Partial hepatectomy was done according to the procedure of Higgins, G. M. and Anderson, R. M. (1931); 60–75 per cent of the total liver tissue was removed. Freshly prepared solutions of tris (β-chloroethyl) amine HCl (0.6 mg./kg.) (HN3) in saline were injected intravenously on the 2nd, 3rd, 5th and 7th days after operation. Details for these procedures have been described (Ludewig and Chanutin, 1946). Neither the adrenals nor the blood plasma were analyzed in the few animals manifesting jaundice. Partial nephrectomy was done according to a two-stage operation described by Chanutin and Ferris (1932), about 5/6 of the kidney tissue was removed. After an interval of 5 days following the second operation, saline or HN3 was injected intravenously. All operations were performed under pentobarbital anesthesia and the animals were starved after intravenous injection.

The animals were sacrificed by exsanguination from the abdominal aorta while under anesthesia and the adrenals were removed immediately. Clotting

of blood was prevented by potassium oxalate. The plasma cholesterol was determined according to procedures described by Sperry and Brand (1943). The extraction of the adrenals and the procedures for determining cholesterol have been described (Ludewig and Chanutin, 1946).

RESULTS

Partially Hepatectomized Rats—Data for the total adrenal cholesterol contents and plasma cholesterol concentrations and adrenal weights of partially hepatectomized rats injected with saline or HN3 are shown in Table 1. It will be noted at that intervals varying from

TABLE 1. CHOLESTEROL DATA FOR ADRENALS AND PLASMA, AND ADRENAL WEIGHTS OF PARTIALLY HEPATECTOMIZED RATS AFTER INJECTION OF SALINE AND HN3

Days After Inj.	Adrenal Cholesterol Content mg./100 gm. rat		Plasma Cholesterol Concentration mg./100 ml.		Adrenal Weight mg./100 gm. rat	
	Saline	HN3	Saline	HN3	Saline	HN3
Two days after operation						
1	0.40±.04 (8)†	0.48 ±.04 (7)	56±3.6 (7)	56±3.0 (7)	17.0±.95	16.8 ±6.2
2	0.48±.03 (10)	0.36 ±.04 (7)	73±7.1 (10)	54±4.8 (7)	15.6±.50	18.9*± .85
3	0.40±.03 (9)	0.37 ±.05 (10)	44±2.7 (10)	70±6.2* (10)	18.1±.56	22.0*± .90
4	0.55±.04 (9)	0.57 ±.04 (9)	53±2.8 (10)	67±3.8* (9)	18.1±.60	20.5 ± .96
5	0.61±.03 (9)	0.64 ±.07 (9)	53±5.4 (9)	52±4.1 (9)	18.8±.85	23.4*±1.42
Three days after operation						
1	0.60±.04 (7)	0.46*±.03 (10)	47±2.3 (7)	49±2.5 (10)	18.2±.77	16.6 ± .25
2	0.53±.04 (0)	0.41 ±.04 (9)	43±1.9 (9)	51±2.4 (10)	16.7±.48	15.9 ± .90
3	0.64±.02 (11)	0.32*±.06 (10)	43±2.5 (9)	78±5.3* (10)	16.2±.40	20.9*± .66
4	0.63±.03 (11)	0.37*±.06 (8)	53±2.5 (9)	61±5.0 (8)	17.7±.50	24.7*±1.8
5	0.66±.04 (9)	0.64 ±.06 (9)	53±1.2 (8)	55±3.3 (9)	18.9±.70	23.7 ±1.2
Five days after operation						
1	0.48±.02 (10)	0.43 ±.02 (8)	50±2.0 (9)	62±3.7* (8)	14.0±.48	16.0 ± .70
2	0.47±.02 (12)	0.34*±.04 (11)	62±2.5 (9)	62±2.2 (11)	14.3±.38	18.1*± .58
3	0.45±.01 (11)	0.26*±.05 (12)	52±2.3 (9)	76±4.4* (10)	14.1±.51	19.8*±1.50
4	0.51±.14 (10)	0.46 ±.04 (10)	54±3.0 (15)	58±3.7 (10)	15.3±.32	21.3*±1.37
5	0.57±.05 (7)	0.60 ±.03 (10)	41±2.5 (10)	48±2.5 (8)	17.3±.73	19.2 ± .80
Seven days after operation						
1	0.44±.03 (10)	0.42 ±.02 (10)	47±1.9 (10)	62±1.4* (13)	16.1±.30	16.6 ± .41
2	0.42±.04 (8)	0.35 ±.04 (9)	60±4.7 (8)	63±4.2 (12)	15.8±.39	18.8*± .82
3	0.49±.02 (8)	0.28*±.04 (10)	49±3.7 (8)	65±4.9 (9)	16.5±.47	19.7*±1.02
4	0.57±.05 (6)	0.43 ±.04 (9)	57±3.2 (6)	65±3.9 (9)	17.9±.86	18.9 ± .80
5	0.53±.02 (9)	0.63 ±.02 (3)	54±3.4 (9)	60±7.0 (4)	18.3±.81	23.5*±1.30

* Represents significant difference from value of corresponding saline group.
† Figures in parentheses represent number of aimals.

2 to 7 days after operation, groups of animals were sacrificed daily for 5 successive days after injection of saline or nitrogen mustard.

The cholesterol contents of the adrenals of the saline-injected rats are increased similarly to the values for intact rats subjected to starvation (Ludewig and Chanutin, 1946). Injections of HN3 into rats on the 2nd day after partial hepatectomy produces no statistically significant change in the cholesterol content. On the other hand, injections of HN3 on the 3rd, 5th, and 7th days after operation are responsible for decreases in the cholesterol content. This apparent discrepancy could be explained by the fact that the hepatic cells which are regenerating most rapidly on the 3rd day (Higgins and Anderson

1931), (Brues, Drury and Brues, 1936) are probably most sensitive to the cytotoxic effect of HN3 (Gillette and Bodenstein, 1946).

Slight increases in the cholesterol concentration of the plasma are observed after HN3 (Table 1). The adrenal weights per 100 gm. body weight of saline-injected partially hepatectomized rats are increased (Table 1) above the mean value of 11.3 mg./100 gm. body weight for the intact untreated animals. The injection of HN3 into these animals causes a still greater adrenal hypertrophy than is observed in the saline-injected animals.

Partially Nephrectomized Rats—Data for the partially nephrectomized rats are shown in Table 2. The adrenal cholesterol values for

TABLE 2. CHOLESTEROL DATA FOR ADRENALS AND PLASMA, AND ADRENAL WEIGHTS OF PARTIALLY NEPHRECTOMIZED RATS AFTER INJECTION OF SALINE AND HN3

Days After Inj.	Adrenal Cholesterol Content mg./100 gm. rat		Plasma Cholesterol Concentration mg./100 ml.		Adrenal Weights mg./100 gm. rat	
	Saline	HN3	Saline	HN3	Saline	HN3
0	0.71±.03 (11)†		80±2.8 (11)		15.9±0.30	
1	0.59±.05 (8)	0.58±.05 (10)	72±2.9 (9)	68±2.6 (10)	18.8±0.33	21.9*±0.40
2	0.94±.09 (6)	0.52±.05* (9)	63±3.0 (6)	76±2.6 (9)	20.8±0.72	24.2 ±1.30
3	0.61±.02 (8)	0.39+.08* (9)	57±4.0 (7)	76±6.6 (9)	21.3±1.03	25.6*±1.20
4	0.79±.04 (5)	0.54±.06* (8)	55±3.7 (5)	84±7.2* (8)	21.3±0.46	23.9 ±1.12
5	0.84±.04 (9)	0.84±.06 (10)	48±2.7 (10)	72±4.6* (10)	22.0±1.60	27.9 ±1.90

* Represents significant difference from value of corresponding saline group.
† Figures in parentheses represent number of animals.

the saline-injected animals are variable. Injection of HN3 causes a decrease in ester cholesterol content on the 2nd, 3rd, and 4th days; this has been observed in intact animals. The decrease in the palsma cholesterol concentration in the starving saline-injected partially nephrectomized rats is not observed in starving intact animals (Chanutin and Ludewig, 1946). After administration of HN3, the plasma cholesterol concentration remains unchanged and the values are significantly higher than those of the saline controls. The adrenal hypertrophy in these HN3 injected rats is the greatest ever observed in this laboratory (Ludewig and Chanutin, 1946).

SUMMARY

Evidence is presented to indicate that liver insufficiency interferes with the cholesterol metabolism in the adrenals.

No significant changes in the plasma cholesterol concentrations or adrenal hypertrophy can be attributed directly to HN3 in either partially hepatectomized or partially nephrectomized rats.

ACKNOWLEDGMENTS

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INCREASED PROTEIN CATABOLISM IN THYROID-ECTOMIZED RATS: RATES OF URINE UREA EXCRETION AND SERUM UREA CONCENTRATIONS[1]

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UNIFORMITY of opinion does not exist regarding the relationship of hypothyroidism to urinary nitrogen excretion. Magnus-Levy (1907) noted a low nitrogen excretion in myxedematous patients, while Underhill and Hilditch (1909) and Janney and Isaacson (1918) reported no definite change in nitrogen excretion after thyroidectomy. Johnston and Maroney (1939) described negative nitrogen balances in cretins, which could be made positive by the administration of thyroid. Small numbers of subjects were studied by these authors, and their investigations were performed under diverse conditions. This report is presented because no definite conclusion can be drawn from the existing literature on the relationship of hypothyroidism to protein metabolism, and because it was found in the experiments described here that the rate of urea excretion of thyroidectomized rats may increase markedly under certain conditions not associated with a high intake of dietary protein. Urea was measured instead of nitrogen, since these studies were part of an investigation into the factors concerned with total deamination.

METHODS

A total of 152 healthy male albino rats was separated into 14 groups. The number of rats within each group, and the purposes for which they were used are indicated in Tables 1 and 2. Some groups consisted of unoperated controls, some were submitted to mock thyroidectomy, and some were thyroidectomized, using blunt dissection and cauterization of the thyroid bed.[2]

[2] Because it is impossible to remove the thyroid gland of the rat without also excising the parathyroids, both glands were removed at operation. Half of the thyro-parathyroidectomized animals died during the first 2 postoperative weeks with tetanic convulsions. No convulsions were observed in the surviving rats after the second postoperative week. Since measurements of urea excretion were not made until after the third postoperative week, and since existing knowledge of parathyroid function does not relate these glands to protein metabolism except during convulsions after parathyroidectomy (Haden and Orr, 1924), it is not thought that removal of the parathyroid glands influenced the observations reported here.

The thyroidectomized and mock thyroidectomized animals weighed approximately 150 gm. at operation. Three weeks intervened between operation and the first urine collection. As the weight of the thyroidectomized rats averaged 150 gm. 3 weeks after operation, the unoperated controls were selected at equivalent body weights in order to simplify comparison of biochemical and autopsy findings.

In order to avoid alterations in metabolic rates or food consumption due to temperature change (Terroine and Trautmann, 1927; Benedict and Mac-Leod, 1929), the environmental temperature was controlled so that it did not fall below 20° C. The highest temperature recorded was 28° C., and this temperature was reached only on a few occasions for short periods of time. Prior to the urine collection periods, all rats ate the same stock diet containing 17% protein, and were supplied liberally with water. During the time of urine collections, Groups 1, 2, 3, 4 and 6 were kept in special cages designed to measure urine urea excretion and food consumption simultaneously, an excess of stock diet and water was provided, and the food consumption was measured daily. The rats in Groups 7–14 were placed in individual cages during the urine collection period, and were fed a solution of 35% glucose in water with 0.4% sodium chloride and vitamins of the B complex for 4 days before autopsy. Because the unoperated control rats in Groups 1 and 2 ate an average of 12.3 gm. of stock diet daily, and the thyroidectomized rats in Groups 3 and 4 consumed an average of 6.5 gm. of stock diet daily from the time of operation until autopsy, the restricted feeding control Groups 5 and 13 were restricted to an intake of 6.5 gm. of stock diet daily for 24 days.

Urine collections were made at 24 hour intervals, and the specimens were combined to form one urine pool daily for each group. Since rat urine may become contaminated with urease producing organisms, and since urease is ineffective in a sufficiently acid medium, enough acid was added to the urine collection receptacles to insure complete urease inhibition. The animals were killed by exsanguination immediately after the last urine collection, and various autopsy measurements were made. These are reported separately (Persike 1948). Blood serum specimens for rats within each group were pooled. Urea determinations on urine and serum pools were performed by the urease aeration method of Addis (1925).

<div align="center">RESULTS</div>

Growth Rates: The average growth rates are shown by Fig. 1. The average growth rate of the mock thyroidectomized animals was not significantly different from the average growth rate of unoperated controls under similar environmental conditions. Growth of the thyroidectomized rats was arrested immediately after operation, and no essential change in average body weight occurred during the 3 postoperative weeks. The average body weight of the restricted feeding controls progressively declined. Three weeks after the start of restricted feeding, these animals weighed 11% less than the thyroidectomized rats and 30% less than the unoperated controls.[3]

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[3] Others have described a retardation of growth after thyroidectomy in rats (Hammett, 1926) which could be overcome by the administration of dessicated thyroid or thyroxine (Rowlands, 1942). The volume of thyroid tissue present 2 months after subtotal and total thyroidectomy in rats, as determined by serial sections of the neck
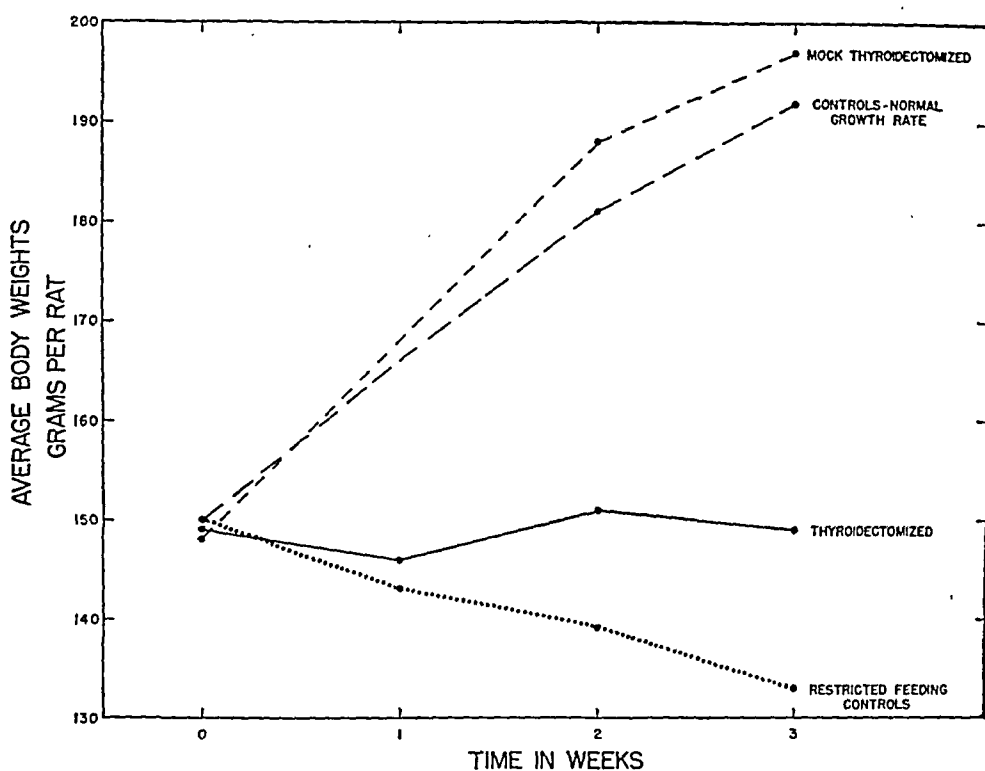
Fig. 1. Average growth rates.

Rates of Urea Excretion: The average rates of urea excretion are given by Fig. 2. Seven days after operation, the thyroidectomized rats excreted 27% less urea than the average of unoperated controls. After this, the rate of urea excretion of the thyroidectomized rats gradually increased, so that 24 days after operation, it averaged 10% less than the control rate. During this time, the unoperated controls ate 12.3 gm. of stock diet per rat daily and the thyroidectomized animals consumed 6.5 gm. of stock diet per rat daily. The restricted feeding control rats, which were given 6.5 gm. of stock diet per rat daily for 24 days excreted slightly less urea at the end of this time than did the freely fed unoperated controls. The mock thyroidectomized animals excreted a little more urea than did the unoperated controls. The mock thyroidectomized rats, however, weighed 30–40% more than the unoperated controls at the time of urine collections, having grown during the 3 weeks after the mock operation (Table 1).

When the diet was changed to 35% glucose solution, the rates of urea excretion of all the animals decreased, but not proportionally.

region, was correlated by Weil (1941) with the degree to which growth was retarded. He found that a reduction in thyroid tissue to one third of normal was sufficient to retard growth, and with lesser amounts remaining the growth rate was proportional to the amount of thyroid tissue removed. When compared with Weil's data, the arrested growth rate during the 3 postoperative weeks of the animals thyroidectomized for the experiments reported here indicates that complete thyroidectomies were performed.
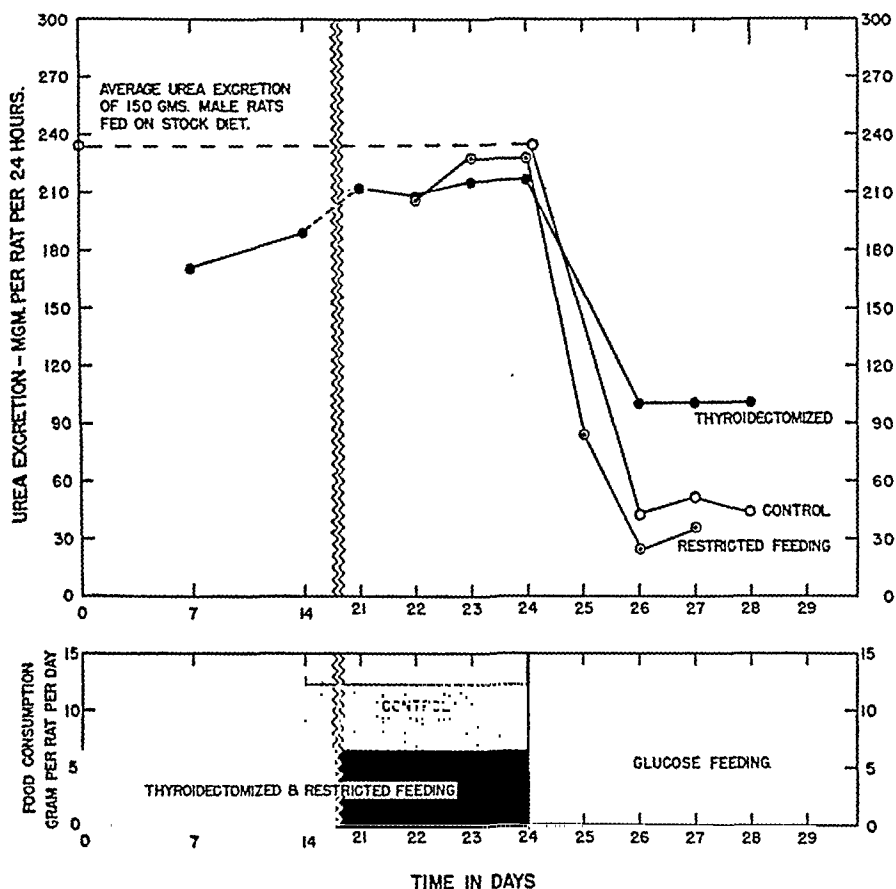
FIG. 2. Average rates of urine urea excretion.

After having been fed glucose solution for 4 days, the unoperated control rats excreted an average of 45.9 mg. of urea per rat daily, while the thyroidectomized animals excreted an average of 96.9 mg. of urea per rat daily, or more than twice the control rate. The rate of urea excretion of the restricted feeding controls was slightly less than the unoperated control rate under these circumstances.

Serum Urea Concentrations: The serum urea concentrations are given in Tables 1 and 2. In all instances, the serum urea concentrations of the thyroidectomized rats were higher than those of the unoperated controls, averaging 44% higher for the rats fed stock diet before autopsy, and 152% higher for the rats killed after 4 days of glucose solution feeding. The serum urea concentrations of the mock thyroidectomized rats were not particularly different from those of the unoperated controls. The average serum urea concentration of the restricted feeding controls which were killed after having been fed stock diet was 34% greater than the average unoperated control concentration, but was not much different than the unoperated con-

TABLE 1. AVERAGE RATES OF URINE UREA EXCRETION AND SERUM UREA CONCENTRATIONS OF RATS FED STOCK DIET BEFORE AUTOPSY

Type of group	Food consumption	Group No.	No. of rats	Av. body wt. at autopsy gm.	Serum urea conc. mg. %			Urea excretion, mg./rat/24 hr.		
					Group	Mean	% change from unoperated controls	Group	Mean	% change from unoperated controls
Unoperated Control	Unrestricted stock diet. Averaged 12.3 gm. /rat/day.	1	12	158	29.0			241.0		
		2	10	156	38.1	33.6		252.6	246.8	
Thyroidectomized	Unrestricted stock diet. Averaged 6.5 gm. /rat/day.	3	11	140	44.9			220.8		
		4	10	146	51.8	48.4	+44	217.3	219.1	−11
Restricted Feeding Control	Restricted stock diet. 6.5 gm./rat/day.	5	9	135	44.9	44.9	+34	221.3	221.3	−10
Mock Thyroidectomized Control	Unrestricted stock diet.	6	11	196	29.7	29.7	−12	280.0	280.0	+12

TABLE 2. AVERAGE RATES OF URINE UREA EXCRETION AND SERUM UREA CONCENTRATIONS OF RATS FED GLUCOSE SOLUTION FOR 4 DAYS BEFORE AUTOPSY

Type of Group	Food consumption	Group No.	No. of rats	Av. body wt. at autopsy gm.	Serum urea conc. mg. %			Urea excretion, mg./rat/24 hr.		
					Group	Mean	% change from unoperated controls	Group	Mean	% change from unoperated controls
Unoperated Control	Unrestricted stock diet followed by glucose feeding.	7	12	131	11.4			40.3		
		8	12	136	9.7			48.4		
		9	12	133	18.0	13.0		49.0	45.9	
Thyroidectomized	Unrestricted stock diet followed by glucose feeding.	10	11	134	38.4			99.0		
		11	9	139	29.4			88.3		
		12	12	130	30.7	32.8	+152	103.4	96.9	+111
Restricted Feeding Control	Restricted stock diet followed by glucose feeding.	13	10	128	11.4	11.4	− 12	30.8	30.8	− 33
Mock Thyroidectomized	Unrestricted stock diet followed by glucose feeding.	14	11	178	9.7	9.7	− 25	59.5	59.5	+ 30

trol concentration for the group which was autopsied after 4 days of glucose feeding.

DISCUSSION

Because the rate of urea excretion depends upon a complex of conditions, there are difficulties in giving a definitive explanation of the behavior of the rates of urea excretion in the thyroidectomized rats. Some of the factors involved in the excretion of urea are the amount of food protein consumed, the rate of protein anabolism which reduces urea formation, the rate of protein catabolism which increases urea formation, the rate of urea formation which depends upon the deamination of amino acids, and the ability of the kidney to excrete urea. A complete understanding of the rates of urea excretion observed in these experiments would require the isolation of each factor. Never-

theless, certain conclusions which have a high degree of probability may be derived from the observations reported here.

An attempt was made to equalize the food consumption effects on urea excretion by restricting normal animals to that amount of food eaten voluntarily by the thyroidectomized rats, since it was found that the thyroidectomized animals ate only half the amount of food which was consumed by the unoperated controls. The restricted feeding controls lost weight progressively on this level of food intake, and it is assumed from this observation that their caloric requirements were not fulfilled. This offers an explanation for the apparent discrepancy between the food protein consumption and the rate of urea excretion of the restricted feeding controls when compared with the unoperated controls while they were eating stock diet. The restricted feeding controls probably used body protein to supply calories, and because of the necessarily increased rate of deamination under these conditions, their rates of urea excretion were increased and their serum urea concentrations rose. When fed glucose solution freely, however, the restricted feeding controls ware able to consume enough calories for energy needs, it was no longer necessary for them to use body protein for this purpose, and their rates of urea excretion and their serum urea concentrations fell to approximately normal levels.

Despite the apparent equality in food consumption, a different situation obtained for the thyroidectomized rats. It is well known that oxygen consumption and carbon dioxide excretion are diminished after thyroidectomy, and from these facts it is inferred that catabolism of carbohydrate and fat are decreased. Fig. 1 shows that the thyroidectomized rats maintained their body weights.[4] Since it seems logical to assume from this finding that their caloric requirements were satisfied, and since they consumed only one half the amount of food protein as the unoperated controls, it might be expected that their rates of urea excretion would be decreased by one half and that their serum urea concentrations would be less than those of the unoperated controls. In fact, however, their rates of urea excretion were only 27% less than the average unoperated control rate one week after operation. Furthermore, their rates of urea excretion gradually increased so that 24 days after operation they were excreting urea at approximately the same rate as the unoperated controls, and their serum urea concentrations were increased by 44% (Table 1). These findings can be explained by an increase in protein catabolism and in the rate of deamination.

An increased rate of protein catabolism is demonstrated more clearly when the rates of urea excretion and the serum urea concentrations of the thyroidectomized rats are compared with the unoperated control values after 4 days of glucose solution feeding, a suffi-

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[4] Hydration of the different groups seemed about the same at autopsy, and an increase in subcutaneous fluid was not observed in the thyroidectomized rats.

cient time to eliminate the influence of the previous stock diet protein consumption. Thirty-five per cent glucose solution is sufficiently concentrated to supply more than enough calories when fed to rats. While no actual measurements were made, there is reason to believe that the thyroidectomized animals consumed enough of this solution to satisfy their caloric requirements.[5] If this is assumed, the strikingly elevated rates of urea excretion and serum urea concentrations of the thyroidectomized animals offer further evidence that protein catabolism and deamination of amino acids is increased after thyroidectomy. Because protein anabolism is reduced to a minimum in rats fed a glucose solution alone, without any dietary protein, these experimental conditions eliminate the possibility that the findings are due to a decreased absolute rate of protein anabolism in the thyroidectomized rats.

SUMMARY

The rates of urea excretion and the serum urea concentrations of thyroidectomized rats were compared with those of appropriate controls, using 2 diets, stock diet containing 17% protein, and a 35% glucose solution. The serum urea concentrations of the thyroidectomized rats were greater than those of the controls on both diets. When fed stock diet, the average rate of urea excretion of the thyroidectomized animals rose from 27% less than the unoperated control rate 1 week after operation, to almost the same as the unoperated control rate 24 days after thyroidectomy. The food consumption of the thyroidectomized rats was one half that of unoperated controls of equivalent body weights. When fed a 35% glucose solution for 4 days, the rate of urea excretion of the thyroidectomized rats was 111% greater than the control rate. These findings are discussed. The hypothesis is advanced that thyroidectomy in rats produces opposite effects on protein metabolism and on carbohydrate and fat metabolism, protein catabolism being increased, and the catabolism of carbohydrate and fat being decreased.

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[5] Attempts were made to measure consumption of the 35% glucose solution by weighing the feeding bottles. The solution was so heavy, however, that leakage prevented measurements from being performed accurately.

INCREASED PROTEIN CATABOLISM IN THYROIDECTOMIZED RATS: ANATOMICAL CHANGES[1]

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WHILE many experimental studies have been made of anatomical changes which occur in hyperthyroid states, few similar investigations of hypothyroidism have been undertaken. Organ weight changes after thyroidectomy have been described, but observed organ weights have not been related to any standard of reference for comparison. The relationship of food consumption to organ weights is well known, but no study of hypothyroidism, with the rat as the experimental animal, could be found in which actual measurements of food consumption had been made, although it has been stated that food consumption of rats probably is decreased after thyroidectomy (MacKay and MacKay 1931; Addis, Karnofsky, Lew and Poo 1938).

In the preceding paper (Persike 1948), data are given to show that the thyroidectomized rats used in these experiments voluntarily ate one half the amount of stock diet which was consumed by unoperated controls of equivalent body weights. On this level of food intake, the thyroidectomized animals maintained their body weights, but normal rats restricted to this level of food consumption progressively lost weight. On the basis of these observations and because of changes found in the rates of urine urea excretion and in the serum urea concentrations, the hypothesis was advanced that protein catabolism is increased in thyroidectomized rats, and that this increase is not to be explained by supposing an inadequate caloric intake. Support for this hypothesis is given by the organ weight changes after thyroidectomy, presented here.

METHODS

The experimental conditions have been given in the preceding paper (Persike 1948). Since the average body weights of the different groups of rats varied because of conditions inherent to these experiments, the organ weights of all the animals were submitted to analysis (Walter and Addis 1939), and are presented both as observed weights and as predicted weights for 150 gm. male rats in Tables 1–3. Serum protein concentrations were

measured from pooled sera by a modification of the biuret method of Kingsley (1939).

RESULTS

Organ Weight Changes of Rats Fed Stock Diet Before Autopsy: Since the mock thyroidectomized rats continued to grow after operation, and were larger when killed than the unoperated controls selected 4 days before autopsy at body weights of 150 gm., the observed organ weights of the mock thyroidectomized animals were greater than those of the controls. When these organ weights were corrected for body

TABLE 1. AVERAGE AUTOPSY FINDINGS (PER RAT) OF RATS FED STOCK DIET WITHOUT RESTRICTION

	Unoperated control			Mock thyroidec- tomy	Thyroidectomized			
	Group 1	Group 2	Mean Groups 1-2	Group 6	Group 3	Group 4	Mean groups 3-4	% change (compared with un- operated control groups 1-2)
No. of rats	12	10	Total 22	11	11	10	Total 21	—
Observed data								
Body Wt. Gm. (at autopsy)	158	156	157	196	140	146	143	− 9
Carcass Wt. Gm.	118	116	117	151	107	111	109	− 7
Liver Wt. Mg.	7542	7557	7550	8352	5964	5983	5974	−21
Heart Wt. Mg.	608	588	598	749	468	451	460	−23
Kidney Wt. Mg.	1127	1122	1125	1285	921	934	928	−17
Abdominal Fat Pads, Mg.	834	1002	918	1619	375	377	376	−59
Drawn Blood Volume, Cc.	5.6	5.5	5.6	6.3	4.6	4.8	4.7	−16
Serum Protein Conc., Gm. %	5.75	6.09	5.92	6.51	6.31	7.09	6.70	+13
Organ Weights Corrected to 150 Gm. Body Wt.								
Body Wt. Gm.	150	150	150	150	150	150	150	± 0
Carcass Wt. Gm.	112	111	112	115	115	114	115	+ 3
Liver Wt. Mg.	7225	7296	7261	6665	6327	6107	6217	−14
Heart Wt. Mg.	585	570	578	615	493	454	474	−18
Kidney Wt. Mg.	1088	1099	1094	1065	969	952	961	−12
Drawn Blood Volume, Cc.	5.3	5.2	5.3	4.9	4.9	4.9	4.9	− 8

weight differences, however, no significant deviations from the organ weights of the unoperated controls were found.

The viscera of the thyroidectomized rats uniformly weighed less than the unoperated control organs. Averages of weights corrected to a body weight of 150 gm. demonstrated a reduction in liver weight of 14%, in heart weight of 18% and in kidney weight of 12%. The corrected carcass weights were 3% heavier than those of the unoperated controls, although a decreased amount of fat was seen at autopsy, and the abdominal fat pads weighed 376 mgm. as compared with 918 mgm. in the unoperated controls (Table 1).

A more marked reduction of fat in the restricted feeding controls was noted, and their abdominal fat pads weighed 144 mgm. The livers of these animals were even smaller than those of the thyroidectomized rats, the corrected liver weights averaging 29% less than the corrected liver weights of the unoperated controls. In contrast to the considera-

ble changes in heart and kidney weights observed in the thyroidectomized rats, the corrected heart and kidney weights of the restricted feeding controls were only 5% and 6% less than the corrected weights of similar unoperated control organs (Table 2).

TABLE 2. RESTRICTED FEEDING CONTROLS (AVERAGE AUTOPSY FINDINGS PER RAT)

	Group 5*	% change (compare unoperated control groups 1–2)	Group 13†	% change (compare unoperated control groups 7–9)
No. of rats	9	—	10	—
Observed Data				
Body Wt. Gm. (at autopsy)	135	−14	128	− 4
Carcass Wt. Gm.	106	− 9	101	− 2
Liver Wt. Mg.	4698	−38	5277	− 7
Heart Wt. Mg.	518	−13	512	− 7
Kidney Wt. Mg.	976	−13	870	− 8
Abdominal Fat Pads, Mg.	144	−84	355	—
Drawn Blood Volume, Cc.	5.0	−11	4.1	−11
Serum Protein Conc., Gm. %	—	—	4.92	+ 4
Organ Weights Corrected to 150 Gm. Body Wt.				
Body Wt. Gm.	150	± 0	150	± 0
Carcass Wt. Gm.	119	+ 6	119	+ 3
Liver Wt. Mg.	5148	−29	6027	− 4
Heart Wt. Mg.	560	− 3	574	− 5
Kidney Wt. Mg.	1053	− 4	971	− 6
Drawn Blood Volume, Cc.	5.6	+ 6	4.8	− 8

* Group 5 restricted to 6.5 gm. of stock diet daily per rat for 24 days.
† Group 13 restricted to 6.5 gm. of stock diet daily per rat for 24 days, following which 35% glucose solution was fed for 4 days.

Organ Weight Changes of Rats Fed 35% Glucose Solution For 4 Days Before Autopsy: When the food of rats is changed from stock diet to a glucose solution containing an adequate content of fluid, calories, salt and vitamins, an absolute loss of body and carcass weight ensues, and an even greater reduction in liver, heart and kidney weight takes place. The specific effects of thyroidectomy still are apparent, however, when organ weights of thyroidectomized rats are compared with those of controls subjected to the same conditions.

The organ weights of the mock thyroidectomized animals were within the bounds of normal variation. The organs of the thyroidectomized rats again weighed less than those of the unoperated controls, the differences being even more marked than those found for the animals fed stock diet before autopsy. Average corrected weights showed the livers to be reduced by 18%, the hearts by 20% and the kidneys by 21%. Hearts and kidneys slightly smaller than normal were found in the restricted feeding controls, the changes being of the same order of magnitude as those previously noted. Instead of a 29% reduction

TABLE 3. AVERAGE AUTOPSY FINDINGS (PER RAT) OF RATS FED STOCK DIET WITHOUT RESTRICTION FOLLOWED BY 4 DAYS OF FEEDING 35% GLUCOSE SOLUTION

	Unoperated control				Mock-thyroid-ectomy	Thyroidectomized				% change (compared with un-operated control groups 7–9)
	Group 7	Group 8	Group 9	Mean groups 7–9	Group 14	Group 10	Group 11	Group 12	Mean groups 10–12	
No. of rats	12	12	12	Total 36	11	11	9	12	Total 32	—
Observed data										
Body Wt. Gm. (at autopsy)	131	136	133	133	178	134	139	130	134	+1
Carcass Wt. Gm.	101	104	104	103	140	104	109	101	105	+1
Liver Wt. Mg.	5772	5856	5435	5688	6796	4635	4813	4654	4701	−18
Heart Wt. Mg.	565	555	536	552	668	444	448	442	445	−19
Kidney Wt. Mg.	966	951	933	950	1188	746	790	748	761	−20
Drawn Blood Volume, Cc.	4.7	4.7	4.4	4.6	5.8	4.1	4.3	4.2	4.2	−9
Serum Protein Conc., Gm. %	4.64	4.74	4.74	4.71	4.88	6.00	5.94	5.84	5.93	+26
Organ Weights Corrected to 150 Gm. Body Wt.										
Body Wt. Gm.	150	150	150	150	150	150	150	150	150	±0
Carcass Wt. Gm.	116	115	117	116	117	117	118	116	117	+1
Liver Wt. Mg.	6494	6358	6017	6290	5869	5122	5120	5265	5169	−18
Heart Wt. Mg.	625	595	585	602	588	483	475	492	483	−20
Kidney Wt. Mg.	1079	1016	1014	1036	1050	808	835	827	823	−21
Drawn Blood Volume, Cc.	5.4	5.2	5.0	5.2	4.8	4.6	4.6	4.8	4.7	−10

in corrected liver weight, however, the average corrected liver weight of these animals killed after 4 days of glucose feeding was only 4% less than the average corrected liver weight of the unoperated controls.[2] The corrected absolute organ weight changes for all the rats are given in Table 4.

TABLE 4. ABSOLUTE ORGAN WEIGHT CHANGES CORRECTED TO 150 GM. BODY WEIGHT OF THYROIDECTOMIZED RATS AND RESTRICTED FEEDING CONTROLS COMPARED WITH UNOPERATED CONTROLS

Average findings gm./rat	Rats fed stock diet before autopsy (Groups 1–6)				Rats fed glucose solution before autopsy (Groups 7–14)			
	Restricted feeding	Thyroidectomized	% change		Restricted feeding	Thyroidectomized	% change	
			Restricted feeding	Thyroidectomized			Restricted feeding	Thyroidectomized
Carcass	+7.000	+3.000	+ 6	+ 3	+3.000	+1.000	+3	+ 1
Heart	−0.018	−0.104	− 3	−18	−0.028	−0.119	−5	−20
Kidney	−0.041	−0.133	− 4	−12	−0.065	−0.213	−6	−21
Liver	−2.113	−1.044	−29	−14	−0.263	−1.121	−4	−18
Drawn Blood Volume	+0.300	−0.400	+ 6	− 8	−0.400	−0.500	−8	−10

Drawn Blood Volumes and Serum Protein Concentrations: The drawn blood volumes (Lippman 1947) and serum protein concentrations of the mock thyroidectomized rats were within the normal range for control rats of equivalent body weights. The corrected drawn blood volumes of the thyroidectomized rats were decreased by 8% to 10%, and their serum protein concentrations were increased by 13% to 26%. The corrected drawn blood volumes and the serum protein concentrations of the restricted feeding controls deviated somewhat from the unoperated control values, but the differences probably were not abnormal.

DISCUSSION

It might be expected that a decreased need for calories would protect the organ and body tissues of thyroidectomized rats from depletion. While this effect may explain their failure to lose weight on a diminished food intake, the rates of urea excretion and the organ weight changes cannot be accounted for on this basis. Other investigators have reported small hearts, livers and kidneys in thyroidectomized rats (Hammett 1927; Addis, Karnofsky, Lew and Poo 1938; Weil 1941). A comparison of the organ weights given here shows that the hearts and kidneys of the thyroidectomized rats were not only smaller than those of the freely fed unoperated controls, but also were smaller than those of the controls restricted to a food consumption

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[2] The corrected heart and kidney weights of all the rats were submitted to statistical analysis. The chance that the differences between the corrected organ weights were fortuitous was found to be less than 1 in 100 when the organ weights of the thyroidectomized rats were compared with the unoperated controls, and between 1 in 100 and 5 in 100 when the organ weights of the restricted feeding controls were compared with the organ weights of the unoperated controls. The author is indebted to Dr. Horace Gray who kindly performed the statistical analysis of these data.

equal to that eaten by the thyroidectomized rats. These changes, therefore, cannot be explained entirely on the basis of a diminished food consumption. MacKay and MacKay (1931) recalculated kidney weights of thyroidectomized rats given by Hammett (1927), and found that they were considerably below the weights which rats receiving no dietary protein should have. Although the work of the kidney was not measured in these experiments, it does not seem likely that diminished kidney work can account for the small kidneys, since kidney work in the rat is determined mainly by the rate of urine urea excretion, which was not decreased in the thyroidectomized rats (Persike 1948).

In the preceding paper, evidence is presented to show that after thyroidectomy in rats, breakdown of body protein is accelerated and deamination of amino acids is increased, despite a lowered need for calories, and that at least part of this increased protein catabolism is independent of dietary influences. Such an effect also might explain some of the anatomical changes reported here, if it were assumed that the differences between the organ weights of the thyroidectomized rats and the unoperated controls were due to changes in the protein content of the organs. The findings of Addis, Karnofsky, Lew and Poo (1938) offer support for this assumption, for the percentage differences between the organ weights of the thyroidectomized rats and the unoperated controls in these experiments are remarkably similar to the percentage differences between the protein content of the organs of thyroidectomized rats and controls which they obtained by direct measurement. Such a hypothesis also offers an explanation for the seeming discrepancy between the relative changes in growth rates and organ weights and the differences in body fat of the thyroidectomized rats and the restricted feeding controls.

The heart is known to be less susceptible to dietary and metabolic changes than the liver and kidney (Addis, Karnofsky, Lew and Poo 1938). The decreased heart weights of the thyroidectomized rats may be at least partially ascribed to a lowered oxygen consumption, a diminished blood volume and less work. An increased serum protein concentration in thyroidectomized rats also was found by Addis, Karnofsky, Lew and Poo (1938), and has been observed in human hypothyroidism by Deusch (1920). The liver weight changes on the 2 diets are difficult to interpret, since the liver is very sensitive to dietary change, and since the glycogen content of the liver may increase greatly when rats are fed a calorically adequate glucose solution. No such considerations, however, explain the small kidneys found in the thyroidectomized rats.

Further investigation is necessary to find the fundamental cause for the changes in protein metabolism observed in these experiments. The question arises as to whether these changes are mediated through altered function of other endocrine glands following thyroidectomy, or whether they are directly due to the hypothyroidism itself.

SUMMARY

Organ weights of thyroidectomized rats and of appropriate controls were submitted to analysis, and are presented both as observed weights and as predicted weights for 150 gm. male rats, thus correcting for body weight differences. The organs of the thyroidectomized rats uniformly weighed less than similar organs of unoperated controls, and their hearts and kidneys weighed less than these organs of normal rats restricted to a food consumption equal to that eaten voluntarily by the thyroidectomized rats. These findings are discussed. It is suggested that these anatomical findings support the hypothesis advanced in the preceding paper that after thyroidectomy in rats, protein catabolism is increased despite a lowered need for calories, and that at least part of this increase is independent of dietary influences.

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THE RESPONSE OF THE HUMAN FETAL REPRODUCTIVE SYSTEM TO THE ADMINISTRATION OF DIETHYLSTILBESTROL AND TESTOSTERONE PROPIONATE DURING EARLY PREGNANCY[1]

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LILLIE in 1917 described the freemartin and attributed this masculinization of the female twin to the presence of androgenic hormones reaching it from the male twin through the anastomosing circulation in the placenta. Since then numerous investigations have been carried out in an attempt to determine whether or not his conclusions concerning the etiology of the condition were correct.

These experiments for the most part were designed to answer the following questions:

(1) Do fetal gonads normally produce specific sex hormones?

(2) Can the gonads be stimulated to produce such hormones even though they do not do so under normal circumstances?

(3) Is it possible for hormones normally present or those resulting from abnormal stimulation of the fetal gonads to affect the development of the reproductive system?

(4) Is it possible to alter the development of the reproductive tract by the artificial introduction of hormones into the environment in which development of the fetal gonads is taking place?

The last question is the one of particular interest to clinicians. There has been a recent trend toward the use of large doses of female sex hormones in early pregnancy and for the occasional use of androgens. If these substances are capable of altering the normal development of the fetal reproductive system, this fact must be taken into consideration when hormonal therapy is contemplated.

The statement has been made on several occasions that the development of the reproductive system is under the control of hormones arising in the fetal gonads and that pseudohermaphroditism and other abnormalities are a result of the alteration of the fetal gonadal secretions. Moore and his coworkers, however, after careful experiments of their own and after evaluating and correlating the experi-

ments of others concluded that there is no satisfactory evidence proving that the gonads produce a secretion during prenatal life or that they are capable of being stimulated to produce hormones during the period when such hormones might have an effect on the differentiation and elaboration of the reproductive organs.

Almost all workers agree that alteration of the hormonal environment in which the reproductive organs undergo differentiation into male and female may produce some modification of the pattern assumed by these organs. Interestingly enough, however, it has been shown that hormonal action is not specific and that both heterologous and homologous organs are stimulated by the presence of excessive amounts of hormones.

Moore (1944), using the opossum in order that hormones might be given by inunction or injection to pouch young and thus avoid the deleterious effect of hormones on the placenta, made the following observations. Male hormone preparations given to females stimulated the embryonic development of the female sex ducts tremendously but did not affect the ovaries. They induced the presence of a prostate, the anlage of which is not normally present in the female. When given to males, androgens stimulated the prostate, phallus and Cowper's glands and also exercised some stimulating effect on the female components of such males. Female hormones given to males stimulated a response on the part of some of the female components of the reproductive system but the male structures responded almost as well. There was no inhibiting effect on the testes. Female hormones given to females resulted in the precocious development of endometrial glands but development of Wolffian ducts was stimulated instead of being inhibited. This was not followed by any alteration in uterine function after birth.

In experimental animals it thus seems possible for the administration of hormones during the period of sex differentiation to result in some alteration in the form of the reproductive system. These changes do not seem to produce permanent alteration or to interfere |with subsequent function.

It becomes important, nevertheless, to attempt to determine in the human whether the therapeutic use of hormones during pregnancy may be attended by demonstrable changes in the reproductive systems of male or female fetuses. Effects produced in one species are not necessarily similar to those produced in another and the only way to be certain of the effect in the human is to study the human. The following investigation was consequently undertaken.

Sex hormones were given early in pregnancy to fifteen women with normal gestations. Ten received diethylstilbestrol, three received progesterone and two were given testosterone propionate. The fetuses in seven of these patients were available for complete histologic studies (Table 1). None of the patients exhibited an alteration in pregnandiol excretion (Davis and Fugo, 1947).

TABLE 1. FETAL SIZE IN RELATION TO HORMONE ADMINISTRATION

| Case number | Fetus | | | | Hormone | |
	Sex	Weight grams	Length cm.	Menstrual Age (days)	Daily dose	Time of administration from first day of last menstrual period
47-1	F	9S	17.5	114	Diethylstilbestrol 200 mg. orally	day 53–114
46-160	M	160	20.0	128	Diethylstilbestrol 100 mg. orally	day 104–117
					200 mg. orally	day 11S–12S
46-160	M	190	22.0	128	Diethylstilbestrol 100 mg. orally	day 104–117
					200 mg. orally	day 11S–12S
46-116	M	215	22.5	137	Diethylstilbestrol 200 mg. orally	day 104–137
47-46	M	216	22.0	133	Testosterone Propionate 50 mg. intramuscularly	day 105–133
47-2	M	268	23.0	131	Diethylstilbestrol 200 mg. orally	day 85–128
46-575	M	360	27.5	150	Testosterone Propionate 50 mg. intramuscularly	day 121–150

Six of these pregnancies were terminated by hysterotomy because of medical indications and one patient suffered a spontaneous abortion.

Four of the patients on whom therapeutic abortions were performed received diethylstilbestrol, two received testosterone propionate. One of the former patients had twins. Six fetuses were male and weighed from 160 to 360 gms. The one female fetus weighed 98 gms.

The lower portions of the ductus deferentes and ureters together with the seminal vesicles, prostate, bladder and urethra were removed as a single unit from each fetus and were fixed and embedded in their entirety. Sections from fifteen to twenty different levels were stained with hematoxylin and eosin. The testes and epidydimides were also removed and sectioned.

Ten fetuses removed by hysterotomy with weights comparable to those in this experimental group were selected as a control series and sections were prepared from corresponding levels of the genital system.

In no instance was it possible to differentiate grossly the fetus of a mother who had received diethylstilbestrol or testosterone propionate from one whose mother had not received hormonal therapy. The appearance of all structures studied was similar to that described in the standard textbooks of embryology and the organs showed a gradually increasing maturity from the 160 gm. to the 360 gm. fetus. The principal changes were in the increasing size of the urinary bladder

and in the thickness of its musculature, in the increasing branching of the seminal vesicles and in the increase in number of glands present in the prostate. The testes showed little change except that in the largest fetus, in contrast to the others, the interstitial cells were slightly less prominent and connective tissue cells were proportionately somewhat increased.

FIG. 1. 46–538 Fetal weight 271 gm. Control fetus—no hormone administered. Section through the urethra at the level of the colliculus seminalis. The uterus masculinis is placed centrally and a portion of an ejaculatory duct is present on each side. Prostatic glands are most numerous in the posterior and lateral portions. ×35.

FIG. 2. 46–160B Fetal weight 190 gm. Diethylstilbestrol was administered to the mother for 24 days prior to therapeutic abortion. This section was taken at approximately the same level as shown in fig. 1. The general appearance is the same. ×35.

Fig. 3. 47–2 Fetal weight 268 gm. Diethylstilbestrol was administered to the mother for 43 days prior to therapeutic abortion. This section is from a slightly higher level than that shown in figs. 1 and 2. The uterus masculinis is in the posterior portion of the prostatic gland and is flanked on either side by the ductus deferentes. The urethra is in the anterior part and the prostatic glands are all posterior to it. ×35.

FIG. 4. 47–46 Fetal weight 216 gm. Testosterone propionate was administered to the mother for 33 days prior to therapeutic abortion. This section was taken at a level between that shown in figs. 2 and 3. The ductus deferentes are slightly more dilated than in the other sections but are no larger than those found in similar control material. The uterus masculinis lies between them and the apex of the hillock of the colliculus seminalis. Prostatic glands are visible in the postero-lateral portions. ×35.

In the 160 gm. fetus each seminal vesicle originates as a slender stalk srising from the ductus deferens. It broadens out to form a small sacculated area and from this two short projections extend upward. Prostatic glands, tending to group themselves in the postero-lateral quadrants, arise from the urethra at a level extending both above and below the origin of the seminal vesicles from the ductus deferentes. A few glands arise from the anterior aspect of the urethra below the level of the seminal vesicles. The prostatic utricle or uterus masculinis, which is the only portion of the Mullerian system that normally remains in this region in the male, appears entirely normal.

The testes are composed of wide cords of cells separated by prominent masses of large polyhedral acidophilic interstitial cells (cells of Leydig). The cords, which are the anlage of the testicular tubules, have no lumens. They are made up of two varieties of cells. Larger cells with pale staining cytoplasm are found principally at the periphery of the cords and more numerous smaller deeply staining cells make up the remainder.

The structure of the same organs in the 190 gm. twin are identical to those just described except that the seminal vesicles show the beginning of a third branch arising from the sacculated portion.

The 215 gm. fetus is much the same except that three definite branches are present in the seminal vesicles and both anterior and posterior prostatic glands are slightly more numerous. The beginnings of small lumens are present in a few of the testicular tubules.

The 216 gm. fetus is almost identical to the one weighing 190 gm. There seem to be slightly fewer prostatic glands than in the 215 gm. fetus but the seminal vesicles are about the same. The uterus masculinis is the same in all three.

The organs of the 268 gm. fetus show a slight increase in complexity, cross sections of four lumens being visible at some levels of the seminal vesicles. The prostatic glands resemble those of the 216 gm. fetus. The cords and interstitial cells of the testes are similar to the other fetuses. No lumens can be seen in the cords.

The reproductive organs of the 360 gm. fetus exhibit the expected slight increase in maturity over the others. Cross sections of the seminal vesicles show six lumens at some levels. The glands of the prostate do not appear to be increased in number. The uterus masculinis is somewhat more irregular in shape than in the younger fetuses but a similar pattern is present in some of the control material. The interstitial cells are slightly less prominent and the connective tissue cells are proportionately more numerous than in the younger fetuses, but this appearance too is found in control fetuses of comparable weight.

The one female fetus weighs 98 gms. and the genital organs are normally developed. No remnants of the Wolffian ducts are visible. The uterus is a single hollow tube surrounded by immature muscle cells and lined by a single layer of cells showing no rudiments of glands.

The cervix is lined by immature squamous epithelium and merges gradually with the vagina. The lower portion of the vagina is without a lumen and is composed of a solid epithelial mass. The fallopian tubes are lined by cells similar to those lining the uterus and have large, well developed lumens. The ovaries are composed of almost solid masses of cells similar to those found in the cords of the testes. Few stroma cells are visible.

CONCLUSIONS

The administration of diethylstilbestrol or testosterone propionate to mothers early in pregnancy in the amounts used, and for the length of time given in this study did not produce any demonstrable changes in the appearance of the gonads or genital organs of 6 male fetuses. It can be concluded that these drugs administered in therapeutic doses early in pregnancy will not affect adversely the development of the genital organs of the human male fetus. The material is inadequate for conclusions concerning the female fetus although the one fetus examined was normal.

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THE ASSAY OF ADRENOCORTICOTROPHIC HORMONE BY THE ADRENAL ASCORBIC ACID-DEPLETION METHOD[1]

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THE ADMINISTRATION of pituitary adrenocorticotrophic hormone (A.C.T.H.) is followed by a temporary decrease in the amount of ascorbic acid present in the adrenals. Under suitable experimental conditions the degree of depletion of adrenal ascorbic acid is a function of the dose of A.C.T.H. administered. This relationship has been adapted to the quantitative assay of adrenocorticotrophic activity (Sayers and Sayers, 1946). The present paper is a detailed description and evaluation of the method based upon the results of a number of comparative assays carried out over the past two years.

DEVELOPMENTAL STUDIES

The adrenocorticotrophic activity of the adenohypophysis is subject to marked change with alterations in the internal or external environment of the organism. For this reason the adrenocorticotrophic activity of materials other than highly purified preparations of A.C.T.H. cannot be assayed in the animal whose hypophysis has not been removed. As will be shown in another section of this paper, even under the most favorable circumstances, i.e., when highly purified preparations of A.C.T.H. are assayed, the response of the intact rat is much more variable than that of the hypophysectomized animal. Urine specimens which showed no activity when tested in hypophysectomized rats produced a definite reduction in the ascorbic acid content of the adrenals of intact rats. Furthermore, urine specimens containing small quantities of A.C.T.H. gave a greater response in intact rats than was warranted by the actual hormone content. On the other hand, the ascorbic acid concentration of the adrenals of the hypophysectomized animal is not influenced by environmental change or administration of a noxious agent and is affected only by the ad-

ministration of an exogenous supply of A.C.T.H. In order to be specific, an assay method for A.C.T.H. must be based on the use of hypophysectomized animals.

When the pituitary is absent, each animal may serve as its own control. The concentration of ascorbic acid in the left adrenal has been found to be equal to that in the right, either when both adrenals are removed simultaneously or when one is removed one hour after the other. In the technic to be described the left adrenal is removed and its concentration of ascorbic acid is taken as the control level from which to measure the amount of depletion produced in the right gland by subsequently administered A.C.T.H.

Following the administration of A.C.T.H. the ascorbic acid content of the adrenal decreases rapidly, remains at a minimum for some time, and then returns to or exceeds the original level. A study was made to determine that period over which the concentration of ascor-

TABLE 1. RESPONSE TO A.C.T.H. IN RELATION TO THE TIME ELAPSED BETWEEN HORMONE ADMINISTRATION AND REMOVAL OF THE ADRENALS

Dose of A.C.T.H. (La-1-A) in micrograms per 100 gm. body weight	Depletion of adrenal ascorbic acid in mg. per 100 gm. fresh tissue			
	30 minutes	60 minutes	90 minutes	120 minutes
0.6	26.5 ± 10.7*	38.5 ± 14.0	50.0 ± 14.2	39.0 ± 16.6
2.4	132.0 ± 7.2	121.5 ± 10.3	109.5 ± 9.2	107.0 ± 12.2

* Average and standard error of four animals.

bic acid is maintained at a minimum following the administration of A.C.T.H. Least variation in response may be expected during this interval. It is apparent from the data presented in Table 1 that the concentration of ascorbic acid in the adrenals is depleted to a level which may be regarded as constant, within the limits of error of the method, over the period extending from 30 to 120 minutes after hormone injection. Two doses of A.C.T.H., 0.6 and 2.4 micrograms per 100 gm. of body weight, were injected into groups of rats and the responses determined 30, 60, 90 and 120 minutes after injection. Treatment of the data by the method of analysis of variance failed to show any significant difference between the responses at the stated times for either of the two doses. The interval of 60 minutes, which is used in the method as finally developed, may be expected to give a minimal variation of response since it lies in the midst of that time period following hormone administration in which the concentration of ascorbic acid is minimal and relatively constant. Previous studies have indicated that it is inadvisable to choose intervals of less than 30 minutes or greater than 120 minutes.

Following hypophysectomy the adrenal cortex undergoes atrophy and may be expected to become progressively less responsive to trophic hormone. For this reason it was considered wise to use the test

animal within as short an interval of time as possible after removal of the pituitary. An experiment was designed to test the influence of variations in the time elapsed after hypophysectomy upon the response to hormone. Eighteen rats were hypophysectomized and divided into three groups. At the end of 18 hours group 1 was given a graded series of doses of A.C.T.H. Group 2 was similarly treated at 24 hours and group 3 at 30 hours after the operation. The log-dose response curves were determined for the three groups and found to be similar. The hypothesis that the three curves were in fact part of the same regression line was tested by the method of analysis of variance (Tippet, 1937) and was not disproved. This would tend to indicate that the response of the animal to A.C.T.H. is relatively independent of the time after hypophysectomy between the limits of 18 and 30 hours. However, the data were not sufficiently extensive to rule out small differences and it is therefore advisable to administer hormone at a fairly uniform time after operation. For reasons of convenience 24 hours plus or minus three hours has been chosen as the time interval.

TABLE 2. ASCORBIC ACID CONCENTRATION OF ADRENALS OF UNTREATED
HYPOPHYSECTOMIZED RATS

Adrenal ascorbic acid total in micrograms			Adrenal ascorbic acid mg. per 100 gm. fresh tissue		
Left	Right	Difference (per cent of left adrenal)	Left	Right	Difference (per cent of left adrenal)
53.0	49.5	6.6	479	479	0.0
49.4	48.4	2.0	477	482	1.0
58.4	56.1	3.9	522	516	1.1
54.0	52.9	2.0	435	435	0.0
52.8	48.3	8.5	443	437	1.4
61.8	63.7	3.1	462	473	2.4
		4.35 ± 1.07*			0.98 ± 0.37*

* Average and standard error.

The response to A.C.T.H. is expressed with the smallest variance as the drop in ascorbic acid per unit weight of adrenal tissue. Since in untreated hypophysectomized rats the concentration of ascorbic acid in the left adrenal equals that in the right adrenal (see Table 2), the depletion produced by hormone is expressed as the difference between the concentration in the left adrenal, removed before A.C.T.H. administration, and that in the right adrenal, removed one hour after hormone injection. The *total* quantity of ascorbic acid in the adrenals is a function of the adrenal weight and is subject to considerably more variation than is the concentration of the vitamin per unit weight of adrenal tissue. This statement is borne out by the data in Table 2 which reveal the fact that although the right adrenal is uniformly smaller than the left adrenal the concentrations of ascorbic acid in the two adrenals are identical.

THE METHOD

Technic. Sprague-Dawley male rats were maintained at an environmental temperature of 84 $\pm 1°$ F. for at least seven days prior to the assay. A mixture of "Calf Builder" meal (94 per cent), wheat germ (3 per cent) and dried brewers' yeast (3 per cent) was fed *ad libitum* prior to and following hypophysectomy. Rats weighing 120 to 160 gm. were hypophysectomized by the parapharyngeal approach. At the time of autopsy the sella was examined under a magnification of 2 times for completeness of hypophysectomy.

Twenty-one to 27 hours after hypophysectomy the rats were anesthetized with sodium pentobarbital (4 mg. per 100 gm. of body weight intraperitoneally). The left adrenal was removed and prepared for analysis. The solution to be assayed was then administered via an exposed tail vein. One hour later the right adrenal was removed and prepared for analysis. The response was expressed as the difference in concentration of ascorbic acid in the left and right adrenals.

The excised adrenal was transferred to filter papers and dissected free of fat and connective tissue with the aid of a fine pair of eye scissors and a head lens of twofold magnification. The capsule was kept intact, care being taken to leave no trace of extra-adrenal tissue which could introduce an error in the quantitative analysis of the gland. During this procedure the gland continuously occupied the spot on the filter paper which it initially moistened. If the dissection was prolonged for any reason, it was interrupted and the adrenal returned to the peritoneal cavity to prevent drying of the tissue. After the extraneous tissue had been removed the gland was wrapped in parafilm and weighed to the nearest 0.1 mg. on an analytical balance or, preferably, on a micro-torsion balance. After transferring the adrenal to the extraction medium the parafilm alone was weighed and adrenal weight obtained by difference. The gland was placed in a 15 ml. conical centrifuge tube containing 12 ml. of 4 per cent trichloracetic acid and a few grains of sand. A glass rod, flattened at one end to fit the bottom of the tube, was used to crush and grind the tissue. Norit (0.3 gm.) was added and mixed with the contents of the tube. Duplicate analyses were made on appropriate aliquots of the filtrate by the method of Roe and Kuether (1943) which determines total (reduced plus dehydro-) ascorbic acid. Accuracy of the analytical procedures may be tested by comparing the concentration of ascorbic acid in the left adrenal with that in the right adrenal of untreated hypophysectomized rats. Table 2 presents the results of a series of such analyses. With rare exceptions the concentrations of ascorbic acid in the two glands of the same rat differ by not more than 25 mg. per 100 gm. of tissue.

Approximately 500 micrograms of A.C.T.H.[4], weighed to the nearest 10 micrograms, was dissolved in 9 drops of 0.01 N NaOH, diluted to about 3 ml.

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[4] A.C.T.H. preparation 62AA (0.4 per cent ash, 4.0 per cent moisture and 15.47 per cent nitrogen on an ash- and moisture-free basis) was prepared according to the method of Sayers, White and Long (1943). Relatively large doses of this material produced no response in assays designed to test lactogenic, gonadotrophic, growth, and thyrotrophic activities. Posterior pituitary principle was present in amounts equivalent to 0.1 I.U. per mg. Preparation 62AA behaved as a single component in the Tiselius apparatus and in the ultra-centrifuge. Preparation La-1-A was obtained through the courtesy of Dr. Paul L. Munson of the Armour Laboratories.

with distilled water and transferred with rinsings to a 15 ml. graduated centrifuge tube. The solution was neutralized by the addition of 0.01 N HCl, using phenol red as an indicator, and diluted so that one ml. was equivalent to 96 micrograms of A.C.T.H. This concentrated solution, which was made up freshly for each day's work, was stored in a refrigerator. The dilute solutions were prepared as needed from the concentrated solution by the addition of 0.9 per cent NaCl. As a precaution they were maintained at 0° C. and discarded after standing for a period longer than 2 hours. The various doses of A.C.T.H. were administered in a volume equal to 0.5 ml. per 100 gm. of body weight. The volume injected was calculated on the basis of the weight of the animal at the time of hormone injection.

TABLE 3. DESIGN FOR ASSAY

Day one	Fifteen* rats hypophysectomized from about 10 A.M. to 2 P.M.
Day two	Schedule of administration of hormone: A.M. (1) T (2) S (3) 2T (4) 2S (5) 4T (6) 4S P.M. (7) 4T (8) 4S (9) 2T (10) 2S (11) T (12) S Rats numbered (in parentheses) in order of hormone administration; hypophysectomies performed in this same sequence. T is a dose of test substance which has approximately the same activity as a dose S of a standard preparation of A.C.T.H.
Day three	Ascorbic acid analyses.

* It is advisable to start with more than the minimum of 12 hypophysectomized rats. The extras serve as substitutes for rats dying before the scheduled autopsy time.

Table 3 presents a convenient experimental design and schedule for use in the present method of assaying adrenocorticotrophic activity. This plan may be repeated as a unit, or in part, until the accumulated number of test animals is sufficient to give the desired degree of accuracy of the assay.

Results. The dose-response curve drawn from the data of a number of assays on A.C.T.H. preparation 62AA extending over the period January, 1946 to January, 1947 is presented in Figure 1a. Eighty animals are included in this plot. The average response (dots) for each of the doses used together with the standard deviation (dashed lines) about the line of best fit (unbroken line) are presented. The curve is that of an hyperbola and may be expressed by the equation

$$ Y = \frac{X}{A + BX} $$

where Y = the response expressed as the drop in adrenal ascorbic acid in mg. per 100 gm. of tissue and X = the dose expressed as micrograms of A.C.T.H. per 100 gm. of body weight of rat. A and B are constants.
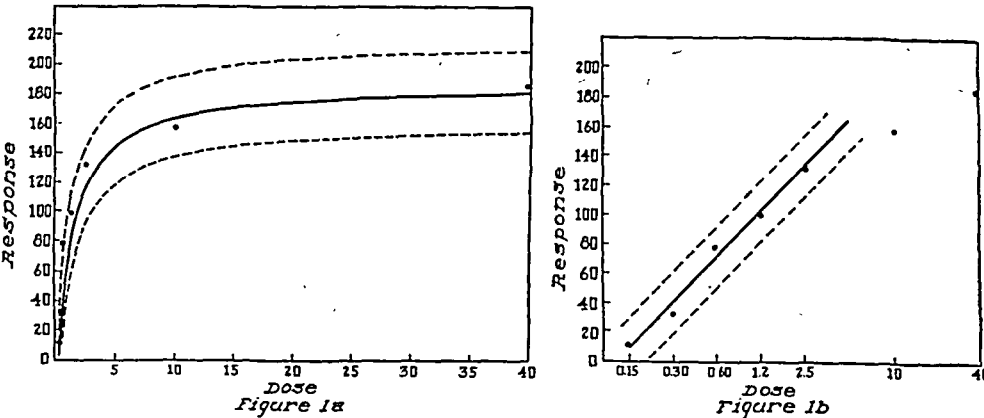
FIG. 1. See text for explanation.

From Figure 1b it can be seen that a rectilinear relation (unbroken line) expressed by the equation

$$y = a + bx$$

where y = the response; x = the log of the dose; a = the y intercept; b = the slope, exists in the dose range from 0.15 to approximately 2.5 micrograms. The dashed lines are located one standard deviation from the unbroken line.

In Table 4 are given the data plotted in Figures 1a and 1b together with the standard deviation of the responses at each dose level.

TABLE 4. STANDARD DEVIATION OF RESPONSE AT VARIOUS DOSE LEVELS OF A.C.T.H.

Dose of A.C.T.H. in micrograms per 100 gm. of body weight	No. rats	Standard deviation of response	Depletion of adrenal ascorbic acid in mg. per 100 gm. fresh tissue
0.15	14	21.5	10.5
0.30	15	24.9	32.3
0.625	16	16.3	77.9
1.25	13	24.2	98.6
2.5	13	15.3	131.1
10.0	6	8.4	156.5
40.0	3	30.5	185.0

Combined standard deviation about means = 20.3.

The standard deviation is almost independent of either the dose or the response level. For all practical purposes the distribution of individual points about the line may be considered to be homoscedastic and therefore standard methods for fitting, comparing and evaluating the errors of the data were used. The mathematical procedures for analysis of assay data have been presented by Bliss (1935a, 1935b), Bliss and Marks (1939a, 1939b), Irwin (1937) and Pugsley (1946), and will not be given in detail here.

The data of a typical assay in which two highly active adrenocorticotrophic hormone preparations are compared together with the per-

TABLE 5. COMPARATIVE ASSAY OF LA-1-A AGAINST 62AA
BY THE DIFFERENCE METHOD

Dose of A.C.T.H. micrograms per 100 gm. body weight	Date Aug. 1947	Concentration of adrenal ascorbic acid (mg. per 100 gm. of tissue)					
		62AA			La-1-A		
		Left adrenal	Right adrenal	Differ-ence	Left adrenal	Right adrenal	Differ-ence
0.6	16	484	441	43	311	287	24
	16	474	444	30	479	396	83
	19	480	457	23	470	451	19
	19	—	—	—	508	479	29
	21	456	433	23	—	—	—
1.2	16	435	373	62	336	239	97
	16	506	422	84	421	360	115
	19	435	377	58	500	463	37
	19	—	—	—	491	448	43
	21	486	409	77	351	250	101
2.4	16	475	363	112	494	345	149
	16	456	350	106	534	341	193
	19	—	—	—	414	257	157
	19	502	371	131	463	330	133
	21	531	355	176	447	304	143

$$
\begin{array}{cccc}
 & 62\text{AA} & \text{La-1-A} & \text{Combined} \\
b = & 168.6 & 195.5 & 182.8 \\
s = & 20.03 & 30.28 & 26.13 \\
\lambda = s/b = & 0.119 & 0.155 & 0.143
\end{array}
$$

$M = 0.0737$; ratio of potency La-1-A/62AA $= 1.185$.
$S_M = 0.0334$; limits of error at $p = 0.95$ are 85% to 117%.
Range of potency La-1-A/62AA in 95 cases out of
$100 = 1.007$ to 1.386.

tinent statistical quantities are presented in Table 5. The doses chosen lie within the limits of the rectilinear portion of the log-dose response curve. The symbols have the following meaning: $b =$ the slope of the log-dose line given by the equation $y = a + bx$, where y is the drop in adrenal ascorbic acid in mg. per 100 gm. of fresh tissue and $x = \log_{10}$ ($10 \cdot$ dose), dose being expressed as micrograms of hormone per 100 gm. of body weight; $s =$ the standard deviation about the line

$$
= \sqrt{\frac{\Sigma(y - Ycalc)^2}{n - 2}}
$$

where Y_{calc} is the value of Y calculated

from the equation $y = a + bx$ and n is the number of observations; λ is the index of precision and is the error of x as estimated from y; M is the logarithm of the ratio of the potency of La-1-A to 62AA; S_M is the standard error of M.

The results of this assay indicate that La-1-A is 1.185 times as potent as 62AA. If the assay were to be repeated 100 times then in 95 cases the ratio of potency of La-1-A to 62AA may be expected to fall within the limits 1.007 to 1.386. It appears that La-1-A is slightly more potent than 62AA.

MUNSON MODIFICATION

Munson, Barry and Koch (1948) have modified the method as described above. One hour after hormone administration both adrenals are removed and combined for analysis. This modification has a distinct advantage in that the number of chemical analyses are reduced to one-half that required by the "difference" method. However, an additional biological variable is introduced, namely; the pre-injection level of vitamin C in the adrenal glands.

Technic. The rats weighed 120 to 160 gm. at the time of hypophysectomy. Twenty-one to 27 hours after the operation they were anesthetized with sodium pentobarbital (4 mg. per 100 gm. of body weight intraperitoneally) and the material to be assayed was administered intravenously. One hour later both adrenals were removed and dissected free of extraneous tissue. The glands were combined, weighed, extracted and analyzed according to the directions given above for the "difference" method.

Results. The response may be expressed as the difference between the concentration of ascorbic acid in the adrenals of untreated controls and the concentration of ascorbic acid in the adrenals of hormone-injected animals. However, when standard and test substances are being compared it is unnecessary to use the level of ascorbic acid in the adrenals of untreated rats as a base line. The values obtained by this method in the hormone treated rats may be used directly as

TABLE 6. COMPARATIVE ASSAY OF LA-1-A AGAINST 62AA
BY THE MUNSON MODIFICATION

Dose of A.C.T.H. micrograms per 100 gm. body weight	Date Aug. 1947	Concentration of adrenal ascorbic acid (mg. per 100 gm. of tissue)	
		62AA	La-1-A
0.6	21	488	400
	21	412	—
	26	409	388
	26	399	464
1.2	21	—	334
	21	—	393
	26	318	383
	26	423	389
	26	361	—
	26	409	—
2.4	21	382	324
	21	336	313
	26	352	299
	26	361	331

	62AA	La-1-A	Combined
$b =$	−115.0	−168.7	−139.9
$s =$	36.88	26.54	32.40
$\lambda = s/b =$	0.321	0.157	0.232

$M = 0.1313$; ratio of potency La-1-A/62AA = 1.353.
$S_M = 0.1017$; limits of error at $p = 0.95$ are 61% to 163%.
Range of potency La-1-A/62AA in 95 cases out of 100 = 0.825 to 2.205.

the responses. When this is done the slope of the log-dose response curve has a numerical value approximately equal to that of the slope of the curve using the "difference" method but is of opposite sign. The rectilinear portions of the log-dose response curves for the Munson modification and the "difference" method cover approximately the same dose range.

Table 6 presents the results of a comparative assay of preparation La-1-A against preparation 62AA by the Munson modification. According to this assay La-1-A is 1.353 times as potent as 62AA and in 95 cases out of 100 the estimate of the ratio of potency of La-1-A to 62AA may be expected to fall within the range 0.825 to 2.205.

ASSAY IN INTACT RATS

An animal with an intact pituitary is of no value for the assay of the adrenocorticotrophic activity of mixtures containing substances which are potential pituitary stimulants. The results obtained are misleading in that they give falsely high values. Furthermore even in the assay of highly purified preparations of A.C.T.H., the variance of the observations is much greater in intact rats than in hypophysectomized animals.

The removal of one adrenal results in a discharge of relatively large quantities of adrenocorticotrophin from the pituitary. For this reason the difference method is not applicable to intact rats.

Technic. Rats weighing 120 to 160 gm. were anesthetized with sodium pentobarbital (5 mg. per 100 gm. of body weight intraperi-

TABLE 7. COMPARATIVE ASSAY OF LA-1-A AGAINST 62AA IN INTACT RATS

Dose of A.C.T.H. micrograms per 100 gm. body weight	Date Oct. 1947	Concentration of adrenal ascorbic acid (mg. per 100 gm. of tissue)	
		62AA	La-1-A
0.6	29	364	256
	29	376	350
	30	—	448
	30	—	341
1.2	29	372	365
	29	327	392
	29	380	429
	30	357	441
2.4	29	248	203
	29	284	305
	29	—	297
	30	316	376
	30	238	—

	62AA	La-1-A	Combined
b =	−181.8	− 88.9	−127.1
s =	33.31	75.10	60.22
$\lambda = s/b =$	0.183	0.845	0.474

$M = 1.8706$; ratio of potency La-1-A/62AA = 0.742.
$S_M = 0.2184$; limits of error at $p = 0.95$ are 35% to 288%.
Range of potency La-1-A/62AA in 95 cases of 100 = 0.260 to 2.137.

toneally). The material to be assayed was administered by tail vein in solution in a volume equal to 0.5 ml. per 100 gm. of body weight. One hour later both adrenals were removed and combined for analysis according to the directions previously presented.

Results. The log-dose response curve obtained in intact rats is of the same character as that obtained in hypophysectomized rats. Table 7 presents the results of a comparative assay of La-1-A against 62AA in intact rats. According to this assay La-1-A is 0.742 times as potent as 62AA. In 95 cases out of 100 the estimated ratio of the potency of La-1-A to 62AA may be expected to fall within the range of 0.260 to 2.137, limits of error much greater than those of either of the two methods of assay in which hypophysectomized rats were employed. Other assays using intact rats have also been characterized by their relatively poor precision.

DISCUSSION

Methods for the assay of A.C.T.H. have employed alterations in adrenal size and in adrenal histology as indices of adrenocorticotrophic activity. For example, in the repair method (Simpson, Evans, and Li, 1943; Sayers, White and Long, 1943) the potency of a preparation is measured in terms of its ability to restore to normal the size and histology of the atrophied adrenals of the hypophysectomized rat. In the maintenance method (Simpson, Evans and Li, 1943; Sayers, White and Long, 1943) activity is measured in terms of the ability of the substance to prevent the atrophy of the adrenals which follows hypophysectomy. The repair method extends over a time interval of 4 days and the quantities of highly purified hormone preparations needed to give a minimal response are in the neighborhood of 10 to 25 micrograms. The maintenance method occupies a period of 14 days and requires a total dose of 70 to 260 micrograms to produce an increase in adrenal size significantly different from that of untreated hypophysectomized controls.

The present method has at least four distinct advantages over these procedures. First, a single injection, rather than multiple injections, is employed. Second, the present technic is much more rapid, the test animals being under the influence of hormone for a period of one hour. Third, the method has a much greater sensitivity, requiring quantities of purified A.C.T.H. of the order of 0.2 microgram to produce a measureable response. Lastly, it has a higher degree of precision. It is difficult to assess the accuracy of the repair and maintenance methods since they have not been subjected to statistical treatment. However, in the authors' experience unexplainable irregularities in response have occurred during the assay of purified preparations of A.C.T.H. by these two methods.

The advantages of the adrenal ascorbic acid-depletion method make it possible to determine the quantity of adrenocorticotrophic

activity in body fluids and tissues. The method is now being applied to the measurement of the adrenocorticotrophic activity of pituitaries, sera and urine of various species.

The method of assay described appears to be specific for A.C.T.H. Evidence has been presented which indicates that the ascorbic acid concentration of the adrenals is under the specific regulatory control of the adrenocorticotrophic hormone of the anterior pituitary (Sayers and Sayers, 1947). Procedures which rapidly and markedly deplete

TABLE 8. PRECISION OF THE METHODS

Date	No. rats	Employing concentration of ascorbic acid in the right adrenal as the response			Employing difference in concentration of ascorbic acid in the two adrenals as the response		
		s	b	λ	s	b	λ
Feb., 1946	10	63.17	−183.9	0.344 (0.299)†	20.01	123.6	0.170
Apr., 1946	15	35.88	− 96.1	0.374	17.85	88.7	0.201
May, 1946	19	25.57	−138.1	0.185	24.47	104.2	0.235
Nov., 1946	12	46.00	−106.7	0.431	23.21	109.0	0.213
Dec., 1946	10	25.71	− 90.8	0.283 (0.152)†	16.23	118.4	0.137
Aug., 1947	12	15.49	−139.5	0.111	20.03	168.6	0.119 (0.076)†
Aug., 1947	14	78.62	−143.5	0.548	30.28	195.5	0.155
				0.325±0.056* (0.279±0.057)†			0.176±0.016* (0.170±0.020)†
		Munson Modification					
Aug., 1947	12	36.88	−115.0	0.321			
Aug., 1947	11	26.54	−168.7	0.157			
Oct., 1947	12	47.83	−203.5	0.235			
Oct., 1947	11	30.57	−283.7	0.108			
				0.205± .047*			

$$s = \sqrt{\frac{\Sigma(y - Y\text{ calc})^2}{n-2}} = \text{standard deviation}$$

$$b = \frac{\Sigma y(x - \hat{x})}{\Sigma(x - \hat{x})^2} = \text{slope}$$

$$\lambda = \frac{s}{b} = \text{index of precision}$$

* Mean and Standard error.
† Values obtained when "aberrant" observations are eliminated by the rule of Sealey and Sondern (1940).[ş]

the ascorbic acid content of adrenals of intact rats, such as bleeding (Sayers, Sayers, Liang and Long, 1945), and administration of epinephrine (Long and Fry, 1945), have no effect on the concentration of the vitamin in the adrenals of hypophysectomized rats. The following substances also gave no response in hypophysectomized rats, when administered in quantities equal to 0.5 ml. per 100 gm. body weight: (1) 5 per cent glucose; (2) 0.9 per cent sodium chloride; (3) extracts (0.5 ml. equivalent to 100 mg. of tissue) of liver, brain and spleen of hypophysectomized rats. This is in striking contrast to the activity of anterior pituitary tissue. An extract of rat adenohypophysis administered in amounts equivalent to 0.10 mg. of this tissue brought about a marked depletion of the vitamin C content of the adrenals. In another experiment A.C.T.H. (62AA) was mixed with a purified preparation of human serum albumin in the ratio of one to 125. Admixture of albumin had no effect upon the potency of the hormone.

Intact rats have an extremely labile endogenous source of A.C.T.H.

[ş] A response is considered abnormal and omitted whenever its contribution to the variance of the mean response exceeds the sum of that for all other individuals in the group.

and are therefore of very little or no value for the assay of adrenocorticotrophic activity. Assays conducted in intact rats are highly variable and non-specific. It is unfortunate that intact rats have recently been employed in attempts to demonstrate the presence of adrenocorticotrophic activity in urine (Blumenthal, 1945; Williamson, 1946).

In Table 8 are presented the standard deviations (s), the slopes (b), and the indices of precision (λ), of a number of assays extending over a period of 18 months. The accuracy of the method employing the difference in concentration of ascorbic acid in the two adrenals as expressed by $\lambda(0.176 \pm 0.016)$ is of the same order as that of the more precise of the biological assay methods. (See Table 3 of the paper by Bliss and Cattell, 1943.)

The influence of the number of test animals employed upon the expected limits of error of the difference method is illustrated in figure 2a. The method of calculation of the limits of error was carried out as follows.

A widely used approximation to the error of M, the log ratio of the potencies, is given by the expression (Pugsley, 1946)

$$S_M = \sqrt{\frac{E^2}{b_c^2} + \frac{(\bar{y}_u - \bar{y}_s)s_{b_c}^2}{b_c^4}}$$

where b_c is the combined slope of the two analyses, $\bar{y}_u$ and $\bar{y}_s$ are the mean responses of the unknown and the standard respectively and s_{b_c} is the standard error of the combined slope.

$$E^2 = s^2\left(\frac{1}{N_u} + \frac{1}{N_s}\right)$$

where N_u and N_s are the number of animals used in tests on unknown and standard and

$$s^2 = \frac{\Sigma(y_u - Y_{u\ calc})^2 + \Sigma(y_s - Y_{s\ calc})^2}{(N_u - 2) + (N_s - 2)}.$$

When the doses of standard and unknown are chosen so that $\bar{y}_u$ approximates $\bar{y}_s$ then the contribution of

$$\frac{(\bar{y}_u - \bar{y}_s)s_{b_c}^2}{b_c^4}.$$

may be disregarded and the expression for S_M reduces to

$$(1) \qquad S_M = \sqrt{\frac{E^2}{b_c^2}} = \sqrt{\frac{s^2\left(\dfrac{1}{N_u} + \dfrac{1}{N_s}\right)}{b_c^2}}.$$

This equation indicates that the error of the ratio of potencies depends upon the variance of the points about the line, the slope of the line and the number of animals used in the analysis. Decreasing the variance, increasing the number of animals or increasing the slope reduce the error.

From accumulated data for the difference method of assay the values of the combined variance and the combined slope were calculated and found to be $s^2 = 490.7$ and $b_c = 114.15$. These estimates were substituted in equation (1) and values of $S_{,f}$ calculated (using $N_a = N$,
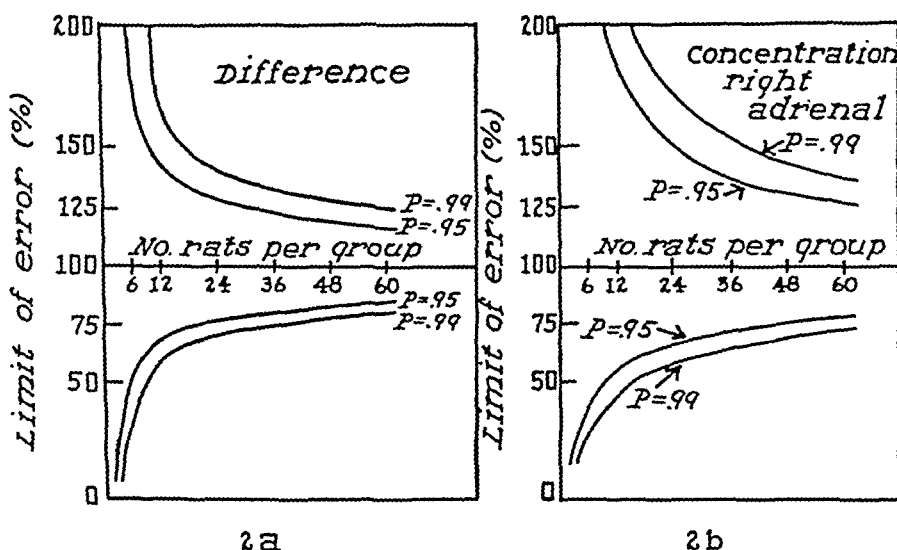
FIG. 2. Relation between the number of rats per group and the expected limits of error of the assay. See text for details.

= number of rats per group) for various numbers of animals. Using appropriate values of "t," the limits of error at the 0.99 and 0.95 levels of probability were then calculated and the results plotted in figure 2a.

A similar calculation was made for the absolute concentration of ascorbic acid in the right adrenal and the results plotted in figure 2b.

From these curves estimates of the number of animals required to give the desired degree of precision of assay can be obtained. Twelve animals per group, that is, twelve on each of standard and test substance have been found to represent a practical balance between the requirements of precision and the labor involved.

The Munson modification has the advantage that the number of chemical analyses required for a given number of rats is reduced to one half. It has the disadvantage that an additional biological variable has been introduced. A limited number of assays have been carried out according to the Munson modification. However, in the instances in which it has been used, although the values of λ have been quite

variable, the average, 0.205 ± 0.047, compares quite favorably with the average value of $\lambda (0.176 \pm 0.016)$ for the difference method (Table 8).

A comparative evaluation of the Munson modification and the difference method may be made from an analysis of difference-method data accumulated to date. In Table 8, values of s, b and λ have been calculated for the same assay data, using in one case the difference in concentrations of ascorbic acid in the two adrenals as the response (difference method) and in the other case the concentration of ascorbic acid in the right adrenal as the response (comparable to the Munson method). It can be seen from Table 8 that the values of s and λ calculated from responses employing concentration of ascorbic acid in the right adrenal are greater and more variable than those calculated from responses using the difference between the two adrenals. In figure 2b, the limits of error of assays in which the concentration of ascorbic acid in the right adrenal was used as response may be contrasted with the values presented in figure 2a, calculated from the same assay data using the difference as the response. It would appear that approximately twice as many animals are required to obtain the same degree of accuracy with the Munson modification as compared with the difference method. A final evaluation of the two methods must await the accumulation of more assay data.

For the *qualitative* detection of adrenocorticotrophic activity in biological material the difference method is the one of choice. The use of the Munson modification involves more labor in such cases since this technic requires a set of untreated controls along with the injected animals, particularly when there is little activity present in the material to be assayed.

Conditions in a particular laboratory, strain of rats, rat diet and a number of unknown variables influence the biological response to most hormones. For this reason the practice of defining the activity of a substance in terms of biological response is to be condemned. Potency of a test substance should, whenever possible, be expressed as the per cent of the potency of a standard calculated from assay data employing test and standard in parallel. Preparation 62AA has been stored in a dry state in a desiccator over calcium chloride. There has been no indication from the assay data accumulated in this laboratory over the past 18 months that this preparation has deteriorated. It is suggested, therefore, that assays of adrenocorticotrophic activity of test substances employ a highly purified preparation of adrenocorticotrophic hormone as a standard.

SUMMARY

Pituitary adrenocorticotrophic hormone depletes the ascorbic acid content of the adrenals of the hypophysectomized rat. This action has been made the basis of a highly specific and sensitive method of

assay of the hormone. The depletion is expressed as the difference between the concentration of ascorbic acid in the left adrenal, removed immediately before hormone injection, and the concentration of ascorbic acid in the right adrenal, removed one hour after the intravenous injection of hormone. A rectilinear relation exists between this depletion and the logarithm of the dose over the range 0.15 to 2.5 micrograms of a highly purified preparation of A.C.T.H. The accuracy of the method is of the same order as that of the more precise biological methods of assay. The average index of precision, λ, for 7 assays has been found to be equal to 0.176 ± 0.016.

It is suggested that a highly purified preparation of A.C.T.H. be employed as a standard against which to compare the potency of test substances.

Intact rats are of little or no value for the assay of adrenocorticotrophic hormone since the responses obtained in these animals are highly variable and non-specific.

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LYMPHOPENIA FOLLOWING ELECTRICALLY INDUCED CONVULSIONS IN MALE PSYCHOTIC PATIENTS

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RECENT animal experimentation has demonstrated that lymphocyte dissolution and lymphoid tissue atrophy is one of the functions of the "S" hormone (11-oxycorticosteroid) of the adrenal cortex (Dougherty and White, 1945; White and Dougherty, 1945; Dougherty and White, 1947). This response is also initiated by pituitary adrenotrophic hormone in the presence of sufficient functional adrenal cortical tissue (Dougherty and White, 1943; Reinhardt and Li, 1945). It is the current opinion that this lymphoid tissue response to pituitary-adrenal cortical secretion is the mechanism responsible for the earlier described thymus and lymphoid tissue changes resulting from various types of stress in experimental animals (Selye, 1937; Ingle, 1938).

There is a paucity of published studies concerning this response in man. One group of workers (Elmadjian and Pincus, 1946; Pincus and Elmadjian, 1946; Hoagland, Elmadjian and Pincus, 1946) have compared diurnal lymphocyte variations and lymphopenia resulting from stress in small groups of normal and psychotic patients. They have concluded that psychotic patients show comparatively anomalous lymphocyte changes in response to the stresses studied. The stress was produced in one group by exposure to an environmental temperature of about 40°C. for one hour and in the other by psychomotor fatigue under moderately anoxic conditions.

In view of these reports, we felt that it might be fruitful to study the lymphocyte response to electrically induced convulsions in man using psychotic patients receiving electric shock therapy. Reported studies of circulating blood cell response to electric shock therapy are few. Jessner and Ryan (1941) state that the white blood count may immediately be doubled during the seizure but shortly returns to normal after cessation of the convulsions. Carse and Slater (1946) performed frequent white cell counts and differentials during a period of one hour following electric shock therapy on psychotic patients and concluded that there was a lymphocytosis reaching its height six minutes following the shock and returning to normal within one

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hour. Centini and di Poggio, quoted from Fin Rud (1947), similarly observed a lymphocytosis during and immediately after electric shock.

METHODS

The subjects used in this study were male patients, all psychotic, selected at random from those receiving electric shock therapy. The standard technic for electric shock therapy used at this hospital was employed. This included the intravenous injection of 0.5 unit curare per pound body weight 5 to 10 minutes before the electrical stimulus in order to minimize the extent of the convulsions. The patients received no sedation or narcosis for 24 hours beforehand. The current used was the minimum necessary to produce a grand mal seizure and varied from 110 millivolts for 0.1 second in some patients to 140 millivolts for 0.4 second in others, the amperage and resistance being kept below 1.5 milliamperes and 1000 ohms respectively.

The 30 examinations reported comprise the results of 18 grand mal reactions, 5 petit mal reactions and 7 controls. A grand mal reaction is designated as representing a generalized convulsive seizure. A petit mal reaction represents the reaction to a subconvulsive stimulus that in all cases resulted in but momentary confusion, flushing of the face, apnea not exceeding 15 seconds and without muscular contractions. In order to obviate sources of emotional stress attendant with the electric shock procedure unshocked controls were included. These controls were subjected to the identical procedures used in administering electric shock therapy to the treated group with the sole exception that no electrical stimulation was given. These controls were chosen from patients undergoing shock therapy and they expected to receive their treatment that day. The routine measures were carried out which included the withholding of breakfast, injection of curare, application of electrodes to the temporal regions of the scalp, insertion of the mouth gag, restraint measures of the 5 attendants and finally the actual flicking of the switch of the shock machine with the electrical current disconnected.

All subjects were examined in the following manner: total white counts were made in the usual manner on oxalated venous blood and differential smears were made from freshly drawn venous blood and stained by Wright's method. Computations of the absolute lymphocyte count were done at 0, 3, 6 and 12 hour intervals following the electric shock procedure. The 0 hour sample of blood, representing the base value, was drawn immediately before the administration of curare which was 5 to 10 minutes before the shock stimulus. Shock was carried out at approximately 9 A.M. and blood samples were subsequently drawn at approximately 12 M, 3 P.M. and 9 P.M. This 12 hour period represents a time of day during which a gradual diurnal rise in lymphocytes usually occurs (Elmadjian and Pincus, 1946).

RESULTS

The results of the examinations of the absolute lymphocyte values in the 30 patients presented here are seen in Table 1, and the representative curves taken as mean values are plotted on Fig. 1. Lymphopenia was commonly present at the 3rd hour following the shock procedure. The values observed at the 6th hour approximated the initial base values whereas the values at the 12th hour were consider-

TABLE 1. ABSOLUTE LYMPHOCYTE COUNTS FOLLOWING GRAND MAL AND PETIT MAL REACTIONS TO ELECTRIC SHOCK IN MALE PSYCHOTIC PATIENTS

Patient no.	Base values before shock	3 hrs. after shock	Percent reduction 3 hrs. after shock	6 hrs. after shock	12 hrs. after shock
Grand Mal Reactions					
1	3080	2431	20	2420	4660
2	3861	2008	48	1856	4550
3	3792	615	84	3346	3832
4	2795	3080	−11	3584	4092
5	2701	1477	49	1737	3120
6	2706	734	75	2368	4625
7	2985	1252	60	4042	4640
8	4095	1770	58	3710	3900
9	1530	1472	7	2660	3159
10	4375	2232	50	3952	3294
11	4162	2360	44	2170	3976
12	2189	1837	19	4079	2800
13	1476	997	33	2702	2875
14	3924	2387	41	3276	4746
15	2170	938	58		
16	2485	1020	59		
17	2423	822	67	2276	
18	2443	2420	0	2379	
Average	2955	1658	45	2909	3876
Petit Mal Reactions					
19	3930	1894	54	3210	4362
20	2618	1292	54	2100	3807
21	3510	1279	52	2492	3900
22	2640	2106	20		
23	2058	924	55	1918	2409
Average	2951	1589	48	2430	3619
Controls					
24	1540	2164	−40	1918	1813
25	3692	3762	− 3	3471	5852
26	2820	3270	−14	3424	3984
27	3429	3850	−11	5642	4900
28	2288	1920	14	3765	4338
29	1782	2835	−55	3078	3877
30	2208	1643	28	3829	5350
Average	2537	2777	− 8	3589	4302

ably higher. The 12th hour samples were taken at a time of day during which lymphocyte counts are usually near their highest. The average 3rd hour figure for 18 examinations in patients following a grand mal reaction showed a decrease of 45% from the original base value, representing a drop of nearly half of the circulating lymphocytes. Two subjects in this series, it is noted, failed to exhibit a lymphopenia. The average 3rd hour value for the petit mal reactors followed very closely those of the grand mal, the figure representing a 48% drop from the base value. Lymphocyte levels conducted at 15 minute intervals on two patients showed this fall in lymphocytes to be a gradual process, the lowest figures being obtained from 2 to 4 hours after

the shock stimulus. Blood samples drawn 15 minutes after the convulsion showed in both cases a distinct lymphocytosis, an observation consistent with that reported by Carse and Slater (1946). The average 3rd hour figure for the controls reveals no lymphopenia and the 6th and 12th hour averages are consistent with a diurnal rise in lymphocytes. Figures for total white counts have not been included, since at the intervals studied they failed to show any constancy. On several subjects the total white counts increased markedly but this increase failed to obscure the 3rd hour lymphopenia. For example, patient #3
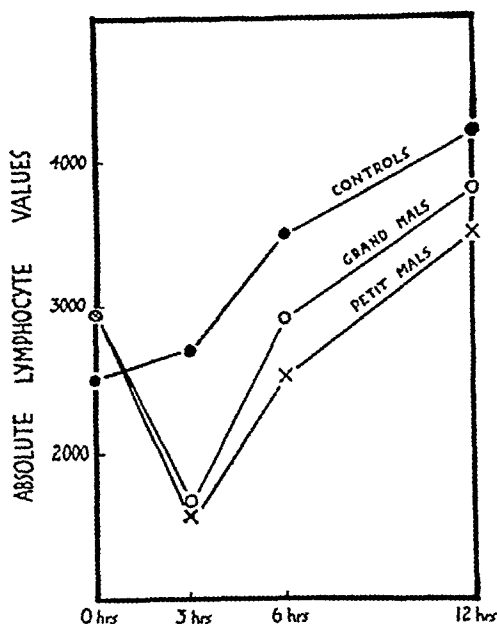
FIG. 1. Graphic presentation of the average absolute lymphocyte values before and at 3 hour, 6 hour, and 12 hour intervals following electric shock in male psychotic patients.

who exhibited the greatest degree of lymphopenia, showed a rise in total white count from 7,900 to 12, 300 at the 3rd hour while the absolute lymphocyte value dropped from 3,792 to 615. Although we were not concerned with the polymorphonuclear leucotytes in this report, it should be mentioned that a moderate degree of neutrocytosis did develop in the majority of instances.

DISCUSSION

This study has demonstrated that an absolute lymphopenia follows electrically induced grand mal and petit mal seizures in male psychotic patients. The time of appearance of this lymphopenia corresponds with the appearance of the lymphopenia reported following the administration of pituitary adrenotrophic or adrenal cortical prepa-

rations and following stress in experimental animals. This suggests that pituitary-adrenal cortical secretions may be responsible for the lymphopenia observed in this series. The manner in which the pituitary-adrenal cortical secretions may be stimulated by electric shock is a matter for conjecture. The possible role of extreme muscular exertion or of hypoxia and hypercapnia is minimized by the occurrence of the lymphopenia following the petit mal seizures. Another possibility is that the anterior pituitary is stimulated either by the electrical current itself or by autonomic fibers. Higher automonic centers could conceivably be stimulated either by direct action of the stimulating current on the hypothalamus or by activation of hypothalamic afferent pathways.

If the lymphopenia studied is due to stimulation of the pituitary-adrenal cortical secretions, patients receiving electric shock therapy present an unique opportunity for further study of this response in man. In addition, animal experimentation using this type of stimulation, may further clarify the mechanism responsible for the lymphopenia.

SUMMARY

Evidence is presented that a significant lymphopenia is found on the 3rd hour following both grand mal and petit mal reactions to electric shock therapy in male psychotic patients. The relation of this finding to the lymphopenia following various forms of stress as well as the administration of pituitary adrenotrophic and adrenal cortical preparations is discussed.

ACKNOWLEDGMENTS

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THE EFFECT OF INANITION ON THE ANTE-RIOR PITUITARY-ADRENOCORTICAL INTER-RELATIONSHIP IN THE GUINEA PIG[1]

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IT IS ABUNDANTLY clear from both clinical studies and those on laboratory animals that malnutrition profoundly modifies the structure and function of endocrine glands, and that these modifications eventuate in hypofunction of the entire endocrine system (Jackson, 1925, 1929, Stephens, 1941). Convincing experimental evidence has been presented, moreover, which indicates that the regressive changes appearing in various target organs, for example, gonad and thyroid, are attributable largely to suppression of appropriate trophic mechanisms in the pituitary of the starved animal (Mason and Wolfe, 1930; Selye and Collip, 1936; Werner, 1939; Mulinos and Pomeranz, 1940; Stephens, 1940; and D'Angelo, Gordon, and Charipper, 1942). A striking and exceptional change in this general pluriglandular deficiency is the hypertrophy of the adrenal cortex which occurs in certain starvation states. The many circumstances in which the adrenals are known to hypertrophy have been collated by Tepperman, Engel, and Long (1943a); and Swann (1940), Ingle (1942), and Long (1947) have reviewed the evidence which establishes the hypertrophic response as being mediated, in most instances, through the adrenotrophic mechanism of the anterior pituitary. Further evidence for the close relationship of the pituitary and adrenal cortex has come from experiments which demonstrate that atrophy of the cortex can be induced in rats by the administration of excess doses of cortical hormone—an effect presumably accomplished through suppression of pituitary adrenotrophic hormone secretion (Ingle, Higgins, and Kendall, 1938; Ingle, 1939; and Wells and Kendall, 1940).

It must be emphasized that practically all studies which relate cortical hypertrophy and atrophy to pituitary regulation have been done in the rat; the need for similar studies on other laboratory forms is urgent (Ingle, 1942). The use of inanition "per se" as a type of stressing agent in adrenal studies, moreover, has been generally avoided despite the fact that some of the stress procedures employed

involve decreased food intake, a factor which in itself influences adrenal morphology. No less important to the elucidation of pituitary-adrenocortical interaction is the problem of anterior lobe activation. The general inhibiting action of starvation on the gonadotrophic and thyrotrophic mechanisms, on the one hand, and the apparent augmentation of adrenotrophic activity, on the other, provide an interesting approach to the elusive problem of anterior pituitary response and the cyto-physiological changes characterizing it. Accordingly, an intensive study is being made of the effect of starvation on the structural and functional interrelationships of the endocrine glands in the guinea pig. Some preliminary observations have already been published (D'Angelo, Gordon, and Charipper, 1941; 1948). The present work is concerned with one phase—the pituitary-adrenal relationship—of this general investigation.

MATERIALS AND METHODS

The immature female guinea pig was used as the experimental animal. The animals came to the laboratory at 225–250 gram body weight, were then placed on the standard laboratory ration of purina chow supplemented with large amounts of greens, and maintained at the usual laboratory temperature (70–80°F.). The body weight was recorded daily. The starvation experiments were begun when the body weight had attained approximately 300 grams (range, 290–320 grams). Only animals which showed a constant weight gain were utilized either for control or starvation regimes.

In the first series of experiments, the effects of acute inanition (no food given, but water "ad libitum") and chronic inanition (general underfeeding) were determined. For acute inanition, animals were starved from 2–9 days. Chronically starved animals were studied over 17–25 days during which each animal received 5–6 grams daily of a basal ration (modified Sherman diet, Stephens, 1940) supplemented with small amounts of greens. All guinea pigs on starvation diets were kept in individual metabolism chambers. Body weight loss, water intake, and urinary output were recorded daily. Animals were sacrificed by anesthesia and exsanguination at various intervals in the starvation period. The endocrine glands were then quickly removed, carefully trimmed, weighed on a torsion balance to the nearest milligram, and then placed in suitable fixatives for subsequent histological and cytological study.

In the next set of experiments, the effect of starvation was tested on the hypophysectomized guinea pig. The operation was accomplished through the parapharyngeal approach and its completeness was subsequently checked by examination of the sella turcica.[1] Hypophysectomized guinea pigs were given small amounts of sweetened tomato juice and lettuce over a period of 1–9 days during which body weight loss occurred. Animals were sacrificed at 1, 2, 3, 8, and 9 days after hypophysectomy and their adrenal gland weight then compared to those in unoperated animals with comparable degrees of body weight loss.

In other experiments, attempts were made to prevent the adrenal enlargement of starvation by the administration of large doses of cortical hormones. Guinea pigs which had been fed normally to approximately 300 grams of body weight were divided into several groups and then subjected to complete starvation (water ad libitum) for 5–7 days, during which various

cortical substances were administered. Individuals of the first group received 1.0–1.5 cc. daily injections of lipo-adrenal extract in oil (Upjohns', 40 rat units per cc.) for 6 days. Members of a second group were given daily injections of 15 mgm. of desoxycorticosterone acetate in saline suspension for 6 days. To guinea pigs of another group were administered 2–15 cc. of aqueous cortical extract (Upjohns', 2.5 rat units per cc.) daily for 4–6 days. A fourth group received .saline control injections. All injections were administered subcutaneously, and at different sites. In all animals except those of the lipo-adrenal group, the injections were given at least twice daily. Animals were sacrificed in most cases on the 7th day of starvation, approximately 18 hours after the last injection.

The effect of cortical administration was also investigated in the normally fed guinea pig. The plan of the experiment was similar to that just described. In this case, however, animals with an initial weight of approximately 300 grams were kept on the normal diet while receiving subcutaneous injections of either aqueous cortical extract (10 cc. daily, for 8 days), lipo-adrenal extract (1 cc. daily, for 10 days), or desoxycorticosterone acetate (15 mgm. daily for 6 days). Control animals were given corresponding amounts of saline. Animals were again sacrificed, as above, at the 18th hour after injection.

<div align="center">EXPERIMENTAL RESULTS</div>

A consideration of the data of table 1 indicates that the immature female guinea pig adequately fed to a body weight of approximately 300 grams, then acutely or chronically starved, displays enlargement of the adrenal glands. It can be seen from the ratio of the adrenal gland weights to the initial body weight of the animal that the hypertrophy of the gland is not merely relative to the decrease in body weight but represents an absolute increase in the size of the adrenal with starvation. The adrenal enlargement is roughly proportional to the degree of body weight loss, and will occur whether the body weight loss is achieved relatively rapidly as in acute starvation or more slowly as in the chronically underfed animal. There is a moderate amount of variation in the rate at which animals lose weight under apparently identical conditions of starvation, and still greater variation in the adrenal weights in guinea pigs with the same degree of body weight loss. Less variability results, however, if the adrenal weights are compared in animals at the same level of body weight loss rather than in those starved for similar periods of time.

The general trend of the adrenal enlargement with progressive body weight loss is shown in figure 1. Despite the limited number of animals presented with approximately 20% loss in body weight the trend of adrenal hypertrophy is apparent at this level.[2] The enlarge-

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[2] It is, thus, apparent why the nutritional history of the animal prior to the experiment should be known. A guinea pig weighing 300 grams which has undergone, for example, an accidental weight loss of 60–70 grams will show adrenal weights not only proportionately high for the apparent initial body weight, but also may have superimposed on it a certain amount of adrenal hypertrophy. Standard weight data relating the endocrine glands to body weight in the growing and mature guinea pig have been given by Deansley and Rowlands (1936) and Mixner, Bergman, and Turner (1943).

TABLE 1. EFFECT OF STARVATION ON THE BODY AND ADRENAL
GLAND WEIGHTS IN THE IMMATURE GUINEA PIG

Treatment	No. animals	Mean body wt. (gm.) Initial	Final	Wt. loss per cent	Mean adrenal (2) wt. (mg.)	Adrenal wt. (mg.) 100 gm. initial body wt.	Mean pituitary wt. (mg.)
Normal Fed	20	313	—	—	133 ± 11* (85–167)	42.2	8.7 ± 1.7 (6–11)
Acute Starvation (2–3 days)	6	317	255	19.7	160 ± 23 (110–200)	50.5	—
Acute Starvation (5–7 days)	9	317	211	33.4	196 ± 28 (151–241)	61.8	7.4 ± 1.0 (6–10)
Acute Starvation (6–9 days)	6	302	176	41.7	220 ± 28. (190–269)	72.1	6.5 ± 1.2 (5–8)
·Chronic Starvation (17–25 days)	7	310	212	31.8	188 ± 52 (116–265)	60.6	—
Hypophy- sectomized. Starved 1 day	2	299	260	13.0	145 ± 18 (127–163)	48.5	—
2–3 days	3	307	234	23.7	137 ± 12 (118–148)	44.6	—
8–9 days	3	306	213	30.4	86 ± 8 (76–98)	28.1	—

* Mean Adrenal Wt. ± average deviation, and in parentheses, the range.

ment of the adrenals is well established at about 30% body weight loss in either acutely or chronically starved animals, and at approximately 40% body weight loss the majority of animals in this category will possess adrenal gland weights exceeding those in the highest range of normal.

That the adrenal gland hypertrophy in starvation depends either directly or indirectly on the presence of the hypophysis is attested to by the failure of hypertrophy to occur in the hypophysectomized guinea pig. Although it is rather difficult to carry hypophysectomized guinea pigs through a starvation regime of any severity, it was found that the adrenal enlargement expected of starved animals with approximately 30% body weight loss did not occur (table 1, figure 1). In fact, the adrenal glands of animals which had been hypophysectomized for 8–9 days were undergoing atrophy as indicated by the low ratio of adrenal to initial body weight. The degree of atrophy encountered was of the same order of magnitude previously described in the mature male guinea pig after long term hypophysectomy (Schweizer, Chari2per and Kleinberg, 1940).

Histological examination of the hypertrophied adrenals in starved animals reveals the enlargement to be the result of an actual increase in cortical mass, and does not involve the medulla. The hypertrophy of the cortex, however, is largely confined to the zona fasiculata, with some contribution by the zona reticularis. In both these zones the cells are swollen and show much vacuolation. Many of the nuclei are dense and hyperchromatic particularly in the reticularis portion. Mitoses are found, usually in the outer portion of the fasiculate zone. The reticular cords tend to separate, and here as well as in the fasiculate region, there is much hyperemia. Many heavily pigmented cells appear. In marked contrast to the hypertrophy of the inner cortical zones is the atrophy of the zona glomerulosa. The spherical cluster-like arrangement of cells which normally characterizes this region is replaced in the starved animal by a narrow, shrunken,

ABSOLUTE HYPERTROPHY OF THE ADRENAL GLAND AS RELATED TO BODY WT. LOSS IN THE STARVED GUINEA PIG

▲ CHRONIC STARVATION
△ HYPOPHYSECTOMY
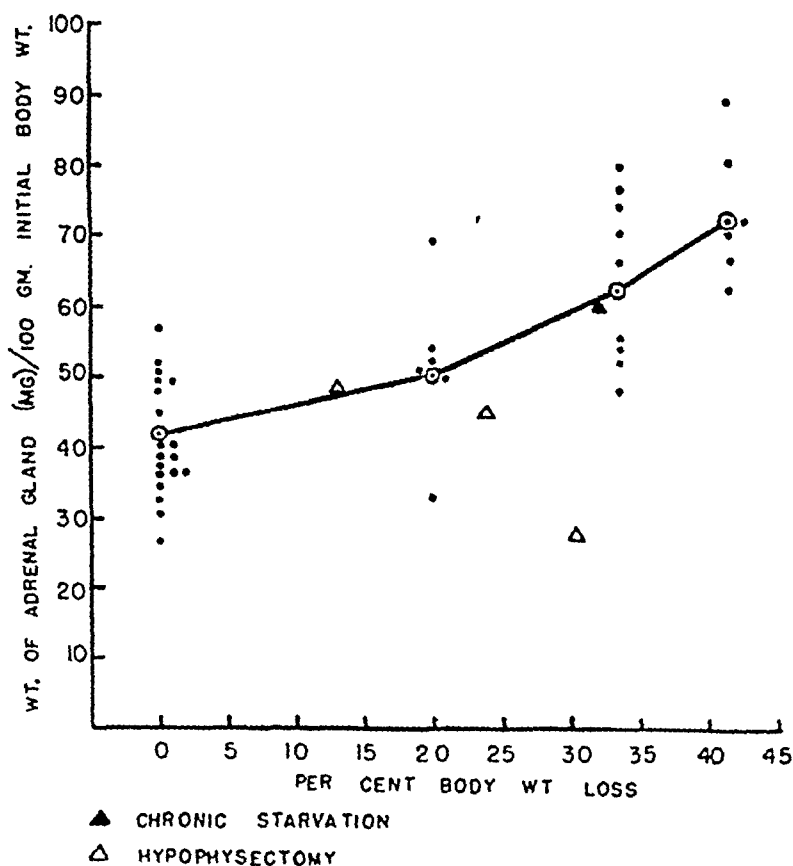
FIG. 1

darkly staining band of cells with scanty cytoplasm and pycnotic, densely staining nuclei. In advanced starvation, it becomes difficult to differentiate the cells of the glomerulosa from the outer capsule, and the hypertrophied cells in the fasiculate zone appear to radiate directly to the capsular region. In general, the structural changes in the medullary region are not as marked as those in the cortex. There is sinusoidal distension in the juxta-medullary region. The cells of the medulla appear to undergo a decrease in size.

TABLE 2. EFFECT OF CORTICAL SUBSTANCES ON TNE PITUITARY AND ADRENAL GLAND WEIGHTS IN THE ACUTELY STARVED GUINEA PIG

Treatment	No. of animals	Days starved	Mean initial body wt. (gm.)	Per cent body wt. loss	Mean adrenal wt. (mg.)	Adrenal wt. (mg.) 100 gm. initial body wt.	Mean pituitary wt. (mg.)
Saline	6	7	304	35.9	235 ±25[1] (207–292)	77.3	7.0 (5–8)
Aqueous Cortical Extract							
2 cc. daily 6 days	2	7	298	33.9	222 ±18 (204–239)	74.5	7.0 (6–8)
5 cc. daily 6 days	2	7	314	37.6	244 ±84 (204–239)	77.7	7.0 (6–8)
10 cc. daily 6 days	3	7	308	33.4	172 ±15 (153–195)	55.9	6.7 (6–7)
15 cc. daily 4 days	2	4*	305	23.6	160 +20 (140–180)	54.5	5.5 (5–6)
Lipo-Adrenal Extract							
1.0–1.5 cc. daily, 6 days	3	7	308	38.7	255 ±7 (245–260)	82.8	7.0 (5–8)
DOCA							
15 mg. daily in saline 6 days	3	7	318	32.4	245 ±67 (178–312)	76.7	7.0 (6–8)

* Both animals found dead on 5th day of starvation.
[1] Refers to Mean Adrenal (2) Wt. ±average deviation, and, in parentheses, the range.

The mean pituitary weight was found to decrease in acute inanition (table 1). The diminution in weight of the pituitary with starvation was not as great as the body weight loss, nor did it appear to correlate well with the hypertrophy of the adrenals. The cytological structure of the gland, however, was greatly altered in starvation. The acidophiles lost their granulation, decreased in size, and became less numerous. There was an apparent increase in the numbers of chromophobes and basophiles, and both of these cell types underwent

enlargement. The basophiles were especially well developed and were present in various stages of degranulation and vacuolation. Osmicated preparations indicated that the hypertrophied basophiles possessed extremely large, arboreal types of Golgi apparatus. In many degranulated basophiles the mitochondrial content was abundant. In contrast, the Golgi apparatus of the acidophiles was always relatively small and compact. The cytological changes just described were progressive and were accompanied also by degenerative changes as starvation became advanced. In the late stages of inanition, the glands were hyperemic and many of the nuclei were pycnotic and hyperchromatic. The sinusoids were distended and contained many hemal elements embedded in a densely basophilic staining coagulum. The specific staining reaction was eventually lost in some starved glands and chromophilic cells were differentiated only with difficulty.

Analysis of the data presented in table 1 indicates that none of the cortical substances administered to the acutely starved guinea pig was able to induce cortical atrophy or prevent appreciably the characteristic hypertrophy of starvation. In all cases, it may be seen that there is a significant increase in the adrenal-initial body weight ratio over that in the fed animal. A comparison of the adrenal response in the liver-adrenal, desoxycorticosterone and aqueous cortical treated groups, as well as in saline animals, shows the adrenal enlargement to be of the same order of magnitude for comparable degrees of body weight loss. Interpretation of the adrenal response in the aqueous cortical group, however, was complicated by the fact that the high doses were not well tolerated. Guinea pigs receiving the 15 cc. daily injections survived only 4 days of starvation so that the efficacy of such large doses in depressing the adrenal weight could not be truly ascertained. It appears significant, nevertheless, that the degree of adrenal hypertrophy found in this group was approximately equal to that found in starved untreated animals at a comparable degree of body weight loss (table 1). The 10 cc. daily dose of aqueous cortical extract was better tolerated, and in this group some tendency for lower adrenal weights was evident. Essentially, however, all of the cortical materials were largely ineffective in preventing the adrenal hypertrophy, and it appears that the relative differences in degree of adrenal enlargement which were found are better explained as differences in response to the added stress of general experimental treatment rather than to any specific effect of the cortical substances "per se." There was no significant change in the pituitary weight of starved animals receiving cortical treatment, nor any appreciable change in the progress of the body weight loss.

That the slight tendency for adrenal depression in the starved animal with aqueous cortical extract may be real rather than apparent is supported by the fact that, of the various cortical principles tested, only the aqueous extract was able to depress significantly the adrenal

TABLE 3. EFFECT OF CORTICAL SUBSTANCES ON THE ADRENAL WEIGHT
IN THE NORMALLY FED GUINEA PIG

| Treatment | No. of animals | Mean body wt. (gm.) | | Mean adrenal wt. (mg.) | Adrenal wt. (mg.) |
		Initial	Final		100 gm. final body wt.
Saline 2–10 cc. daily 6–10 days	16	300	350	163 ±22* (128–205)	46.6
Aqueous Cortical Extract 10 cc. daily, 8 days	3	302	342	122 ±16 (98–138)	35.6
Lipo-Adrenal Extract 1 cc. daily, 10 days	3	314	354	164 ±14 (150–185)	46.3
DOCA 15 mg. daily in saline, 6 days	3	310	317	169 ±11 (153–182)	53.5

* Refers to Mean Adrenal Weight ± average deviation, and, in parentheses, the range.

weights in the normally fed animal (table 3). All three of the animals receiving the 10 cc. daily doses of aqueous cortical extract possessed adrenals whose weights were appreciably below the average of those in the saline control group. In contrast, the lipo-adrenal and desoxy-corticosterone-treated fed animals showed adrenal weights which were essentially the same as those of the control group. Although the number of animals tested is small, statistical evaluation of the results with the aqueous cortical extract indicates that the chances for these adrenal weights to be obtained by random selection of animals in the general population is approximately 1 out of 500. It seems significant also that the adrenal weights in these cortical treated animals averaged below those found in normally fed untreated animals at the same initial body weight.

DISCUSSION

Many accounts in the literature of malnutrition emphasize the ease with which morphologic changes are effected in the adrenal gland in various types of dietary insufficiency. Interpretation of these changes is often difficult since differences in the degree of inanition, as well as certain vitamin deficiencies, markedly influence the response of the adrenals. Thus, complete inanition generally results in adrenal hypertrophy, whereas, chronic underfeeding, at least in the rat, may cause adrenal atrophy (Mulinos and Pomeranz, 1940, 1941; Mulinos, Pomeranz, Lojkin, 1942, and Cameron and Carmichael,

1946). The results of the present experiments on the immature guinea pig indicate that the characteristic response of the adrenal to starvation is hypertrophy and that this occurs whether the inanition be acute or chronic. This apparent discrepancy between rat and guinea pig may well reflect fundamental differences in the vitamin requirements of these rodents. The guinea pig is highly susceptible to vitamin C deficiency, and this lack has been reported by many to cause adrenal enlargement (Lockwood and Hartman, 1933; Quick, 1933, Svirbely, 1935; Murray and Morgan, 1946). The rat, in contrast, is notoriously resistant to lack of vitamin C, and adrenal hypertrophy, as well as cytochemical changes, is associated more with deficiency of various constituents of the Vitamin B group (Deane and Shaw, 1947). No apparent scorbutic changes were present in any of the starved guinea pigs in these experiments; the possibility must nevertheless be allowed that chronic underfeeding may result in a degree of vitamin C deficiency which, however slight, may contribute to adrenal hypertrophy.

The hypertrophic changes in the adrenals of starved guinea pigs involve the cortex and not the medulla. Not all of the cortical zones, however, participate in the hypertrophy. The cortical enlargement is contributed to primarily by the fasiculate zone and, to a lesser degree, the reticularis, whereas the zona glomerulosa is characteristically atrophic. The hypertrophy of the inner zones represents a true increase in cortical mass. The solid content of these enlarged adrenals is significantly increased over normal (unpublished Experiments). These structural changes occur in either the acutely or chronically starved guinea pig, and confirm, in the main, Kojima's (1929) description of the adrenals in complete starvation. The marked atrophy of the glomerulosa, as contrasted with hypertrophy of the inner cortical zones, lends some support to the contention of Swann (1940), Greep and Deane (1946), that the glomerulosa, unlike the fasiculate zone, is autonomous and not under pituitary regulation.

It is now clear from recent inanition studies that proper interpretation of endocrine changes in malnourished animals must consider not only direct effects of starvation on glandular tissues, but also secondary effects in those glands whose structural integrity normally depends on trophic stimulation from the anterior pituitary. The atrophic changes occurring in the reproductive system of starved animals are similar to those in hypophysectomy, are associated with decreased gonadotrophic potency of the pituitary, and are readily repaired in the starved animal by pituitary material (Mason and Wolfe, 1930; Moore and Samuels, 1931; Seyle and Collip, 1936; Werner, 1939; Mulinos and Pomeranz, 1940). The morphologic changes which appear in the starved thyroid are also of a regressive nature, and in both mammal and amphibian are referable to primary alterations in the anterior pituitary (Stephens, 1940; D'Angelo, Gor-

don, and Charipper, 1942). In view of the large body of evidence which demonstrates cortical hypertrophy as being under pituitary regulation, it seems likely that in starvation there is suppression of gonadotrophic and thyrotrophic function and augmentation of the adrenotrophic activity in the hypophysis. The present experiments support this interpretation. Cortical hypertrophy fails to occur in the starved hypophysectomized guinea pig, and the cortices eventually become atrophic. The cytological features of the starved pituitary, moreover, are such as to indicate that some of the basophilic cells are in high secretory activity as shown by their enlargement, degranulation, mitochondrial content, and hypertrophied Golgi apparatuses. This heightened secretory activity is occurring at a time when the gonadal system is in the atrophic state, and the thyroid gland in the resting or inactive condition. These considerations seem to indicate rather strongly that, in starvation, there is a shift in the quality of the hormone production so that secretion of adrenotrophin is augmented at the expense of, or in lieu of, other trophic factors. Confirmation of this hypothesis, of course, awaits experiments which rule out any "hypersensitivity" of the cortex to normal amounts of circulating adrenotrophin, and which will demonstrate the presence of increased amounts of adrenotrophin in both the pituitary and circulating fluids of starved animals.

There is much reason to believe that in the normal animal atrophy of the adrenal cortex, like hypertrophy, reflects some change in the level of adrenotrophic activity in the hypophysis. Ingle and Kendall (1937) first demonstrated that overdosage of rats with cortical hormone induced atrophy of the cortex; a response which could be prevented by the simultaneous administration of adrenotrophic hormone. The cortical regression in the rat has since been confirmed by others using either cortical extracts or crystalline steroids. All cortical substances are not equally efficacious in producing the atrophy. Although cortical extracts are effective (Mackay and Mackay, 1938; Ingle, Higgins, and Kendall, 1938; Ingle 1939), those cortical steroids related to carbohydrate metabolism appear to be especially potent (Wells and Kendall, 1940). Desoxycorticosterone can also induce cortical atrophy provided doses are sufficiently high and treatment prolonged (Selye, 1940; Selye and Dosne, 1942; Carnes et al., 1941, Albert and Selye, 1942, Sarason, 1943, and Castillo and Rapela, 1945), and progesterone has also been reported to have some effect (Clausen, 1940). More recently, Sayers and Sayers (1947) have shown the same order of effectiveness of cortical steroids in preventing the depletion of vitamin C in the rat cortex which ordinarily follows either adrenotrophin administration or the imposition of some stress. All of these results support the generally held thesis that a reciprocal regulatory action exists between the anterior pituitary and adrenal cortex, and that cortical enlargement and regression are the result of augmenta-

tion and suppression respectively, of the adrenotrophin mechanism. It must be pointed out that all of these experiments have been done on the rat. The present work indicates that this concept of reciprocal regulatory action between pituitary and adrenal cortex also applies to the guinea pig. In both forms, hypophysectomy prevents adrenal hypertrophy, and, in both, cortical administration to the normal animal depresses the adrenal cortex. We are at a loss, however, to explain the failure of the lipo-adrenal extract to depress the adrenals as did the aqueous cortical extract. The former is allegedly high in potency for those hormones which affect carbohydrate metabolism and resistance to stress (corticosterone, 11-dehydro-17-hydroxycorticosterone) and which are known to induce cortical atrophy.[3] The amounts of hormone given approximate those which were effective in the rat (Ingle, Higgins, and Kendall, 1938; Wells and Kendall, 1940). The production of cortical atrophy may require more hormone in the guinea pig than in the rat, but this will hardly explain why the aqueous cortical extract was effective in dosages as low as those successfully employed in the rat.

Although the present work provides strong evidence for the involvement of the anterior pituitary in the adrenal hypertrophy of starvation, we were unable to demonstrate unequivocally that cortical administration can block adrenal enlargement in the starving animal. Cortical substances were largely ineffective in this regard. There did seem some tendency for the aqueous cortical extract in the 10 cc. daily dose to depress the adrenal weights in starvation. Unfortunately, high doses of aqueous cortical extract were not well tolerated. Possibly some route of administration, other than subcutaneous, may prove efficacious; however, the decreased water ingestion in starvation appears to obviate the oral route successfully employed by Ingle, Higgins and Kendall (1938). The only effective procedure in producing cortical atrophy was hypophysectomy. The relatively short time taken for the adrenals to regress in the hypophysectomized animal would seem to indicate that the experimental period was sufficiently long for some regression to have occurred had the cortical substances been able to effect "functional hypophysectomy" in the intact animal.

The stress of starvation certainly would be expected to increase cortical hormone utilization, and the possibility remains that in this type of stress much greater amounts of cortical hormone are needed to prevent cortical hypertrophy than are necessary for the fed animal. Albert and Selye (1942) were able to prevent and actually reverse the adrenal hypertrophy caused by estradiol administration with amounts of desoxycorticosterone not greatly in excess of those previously used to effect cortical atrophy in normal rats. Many metabolic upsets occur in the starving animal among which the negative nitro-

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[3] We have since tested the lipo-adrenal extract in the normal female rat. Daily 0.5 cc. injections, given for 8 successive days failed to produce cortical atrophy.

gen balance must certainly be an important one. Tepperman, Engel, and Long (1943b) have suggested that changes in the rate of protein catabolism serve as the stimulus for adrenal hypertrophy. If this is so, restoration of a balanced nitrogen economy may be the only factor which can completely prevent the adrenal enlargement of starvation.

SUMMARY AND CONCLUSIONS

Studies have been made on the female guinea pig to investigate the morphologic responses of the adrenal and pituitary glands, and the pituitary-adrenocortical interrelationship in starvation. Enlargement of the adrenals is a characteristic response of the guinea pig to deprivation of food and occurs either in acute or chronic starvation. The increase in adrenal size is both relative and absolute and is roughly proportional to the degree of body weight loss and the duration of the inanition. The adrenal hypertrophy involves the inner cortical zones, primarily the fasiculate and to a lesser extent the reticularis zones. There is atrophy of the zona glomerulosa in either acute or chronic starvation.

The weight of the pituitary gland decreases with starvation. The diminution is not marked, however, and appears to reflect the general loss in body weight. The microscopic structure of the pituitary is greatly altered in starvation. There is a progressive loss of acidophilia, and an increase in the numbers of basophiles and chromophobes. The basophiles reveal cytological manifestations of high secretory activity (cell hypertrophy, degranulation, abundant mitochondrial content, and hypertrophy of the Golgi apparatuses). In advanced starvation the pituitary glands lose their specific staining reaction and degenerative changes in nucleus and cytoplasm are marked.

The cortical hypertrophy of inanition does not occur in the absence of the hypophysis. The administration of aqueous cortical extract in high doses significantly depresses the adrenal weight in the normally fed guinea pig, but does not effectively prevent cortical hypertrophy in starvation. The pituitary weight and progress of the body weight loss in the starved animal is not appreciably altered with cortical treatment.

These results are interpreted to mean that the cortical hypertrophy in the starving guinea pig results from augmentation of adrenotrophin secretion by the basophiles of the anterior pituitary. The ineffectivenesss of cortical hormones in preventing the adrenal hypertrophy suggests the catabolic reactions in starvation to be such that adrenotrophic secretion either cannot be appreciably inhibited, or that much greater amounts of cortical hormone than those effective in the fed animal are necessary.

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THE EXTRA-RENAL ACTION OF DESOXYCOR-TICOSTERONE: SURVIVAL AND WATER INTOXICATION STUDIES[1]

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THE EXPERIMENTS reported here were undertaken with two aspects of the problem of the extra-renal effects of desoxycorticosterone acetate (DCA) in mind; first, the general one of whether the substance has important actions other than those mediated through the kidney, since there has been disagreement on the point; secondly, an attempt was made to test directly previous suggestions that the protective action of DCA in water intoxication is due in part to factors other than the stimulation of diuresis, i.e., to extra-renal influences.

It is now well established that the 11-oxygenated adrenal steroids have important sites of action in organs other than the kidney. Most of the well-known actions of DCA, however, are on the kidney (Swingle & Remington, 1944). Selye (1940) and Selye and Nielsen (1941) on the other hand, claimed that DCA shows life-extending as well as other effects in nephrectomized rats and mice. This conclusion was criticized by Winkler, Smith and Hoff (1942) on the basis of the fact that Selye had begun treatment before nephrectomy. Winkler and collaborators felt, as a result of their work on dogs, that the pre-treatment had lowered potassium stores before operation and in that way accounted for a greater survival after nephrectomy. Such findings are in keeping with the results of Durlacher and Darrow (1942) and Bondy and Engel (1947) who prolonged survival of their nephrectomized rats by dietary depletion of potassium prior to operation.

In regard to water intoxication Swingle, Remington, Hays and Collins (1941) first stated that there was a "sensitivity to hydration" in adrenalectomized animals, in addition to a deficient diuresis, accounting for their susceptibility to the toxic effects of excess water. Their results imply that both deficiencies were relieved by DCA. This idea has been elaborated and supported by others, e.g., Liling and Gaunt (1945), and Hays and Mathieson (1945). None of these workers however, have demonstrated an effect of DCA on such phenomena under conditions where the possibility of renal contribution to the results was completely excluded.

Therefore, the effects of DCA on the survival of adrenalectomized-nephrectomized rats, and on the response of such animals to excess water were studied; in most of the experiments no hormone was given until after nephrectomy so that the potential complications of pretreatment as emphasized by Winkler and associates were eliminated.

Part of this work has been presented in abstract form (Birnie *et al.*, 1947 and 1948).

METHODS

In the simple survival experiments the right kidney and adrenal were removed at one operation and these same organs removed on the left side approximately one week later. Therapies were begun immediately after the second operation. A similar two-stage operation was used when the kidneys only were extirpated.

In water intoxication tests, four or five doses of water, warmed to body temperature, were administered by stomach tube at hourly intervals. Each dose was calculated to equal 4 cc. per 100 sq. cm. of body surface, and for a rat weighing 185 gms. was 12 cc. Water administration was started two hours after the second kidney was removed. Food (Purina laboratory chow) and water were allowed until the time of the second nephrectomy.

In *Series A* both adrenals and the right kidney were removed at one operation and the animals were maintained on 0.5 mg. of DCA in oil per day for 5 to 6 days. Hormone treatment was then withdrawn and 24 to 36 hours later the second kidney was excised and water tests made.

In *Series B* the right kidney and adrenal were removed at one operation and the left kindey and adrenal after an interval of 5 or 6 days. Treatment was started immediately after the second operation and two hours prior to the water tests. In this way possible complications due to pre-treatment with DCA were eliminated.

In both *Series A* and *B* the animals were divided into two groups matched according to weight for the water tests. Half received 2 mg. of DCA subcutaneously plus 1 mg. intramuscularly just after the second kidney was removed and an additional 2 mg. intramuscularly at the time the first dose of water was administered, making a total dose of 5 mg. of DCA. The other half served as controls and received injections of the peanut oil vehicle in a similar manner.

In *Series C* the animals were treated similarly to those of Series B except that they were fasted 18 hours prior to the water test and were quickly anesthetized with Nembutal one hour after either the third or fourth dose of water in order to make measurements of water absorption and certain blood constituents.

Blood determinations were made on heparinized samples taken directly from the heart immediately after the induction of anesthesia. Methods were as follows: plasma chloride, Peters and Van Slyke (1932); plasma protein, Barbor and Hamilton (1926); hemoglobin, acid hematin read in a photoelectric colorimeter; hematocrit, in triplicate capillary tubes using an air driven centrifuge, Parpart and Ballantine (1943); water absorption, Heller and Smirk (1932); rectal temperatures were measured with a thermocouple and potentiometer.

In *Series D* animals were prepared as in Series B except that only the kidney and not the adrenals were removed. Part of these were treated with

DCA as in Series B, and part received aqueous adrenal cortical extract[2] as follows: in each of 4 doses of water given, 1.5 cc. of extract was substituted for 1.5 cc. of water, thus keeping constant the total volume of fluid given. In previous unpublished experiments it had been found that the effectiveness of cortical extract in protecting against water intoxication was maximal when administered in this manner. Controls received similar amounts of normal saline solution in water, since the cortical extract was made up in normal saline.

RESULTS

Survival studies (Table 1). Adrenalectomized-nephrectomized rats treated with 3 mg. DCA per day, beginning immediately after operation, survived an average of 51 hours. Controls given 0.5 cc of peanut oil (solvent used for DCA) survived an average of 30 hours. Untreated rats from which the kidneys but not the adrenals were removed lived for an average period of 29 hours.

TABLE 1. EFFECT OF DCA ON THE SURVIVAL OF ADRENALECTOMIZED-
NEPHRECTOMIZED RATS

	Number of animals	Average weight	Survival in hours		
			Mean	S.E.	Range
Adrenalectomized-nephrectomized DCA treated	9	131	51	±6.6	28–102
Adrenalectomized-nephrectomized controls	10	128	30	±2.6	18– 42
Nephrectomized, adrenals intact	20	128	29	±0.8	24– 36

Thus it is seen that DCA exerts not only an extra-renal life-extending action, but is more effective in this regard than are the animals' own adrenals.

Relation of body weight to survival after nephrectomy (Figure 1). There are wide discrepancies among reports in the literature as to the survival time of rats after nephrectomy (Ingle and Kendall, 1936; MacKay, Bergman and MacKay, 1937; Hartman and Dubach 1940; Durlacher and Darrow, 1942). Although the weights of animals used by different investigators were not always reported, it is probable that different weights were used. We studied therefore the relation of body weight to survival in animals that were nephrectomized in the usual two-stage operation. It is apparent from Figure 1 that survival after nephrectomy increases markedly with increasing body size. This is the probable basis of the various findings previously recorded.

Water intoxication studies (Series A and B: Table 2, Figure 2). The protective action of DCA against the effects of excess water in the nephrectomized animals was striking. Series A and B responded alike and are considered together. Only 3 of the 21 oil-treated controls re-
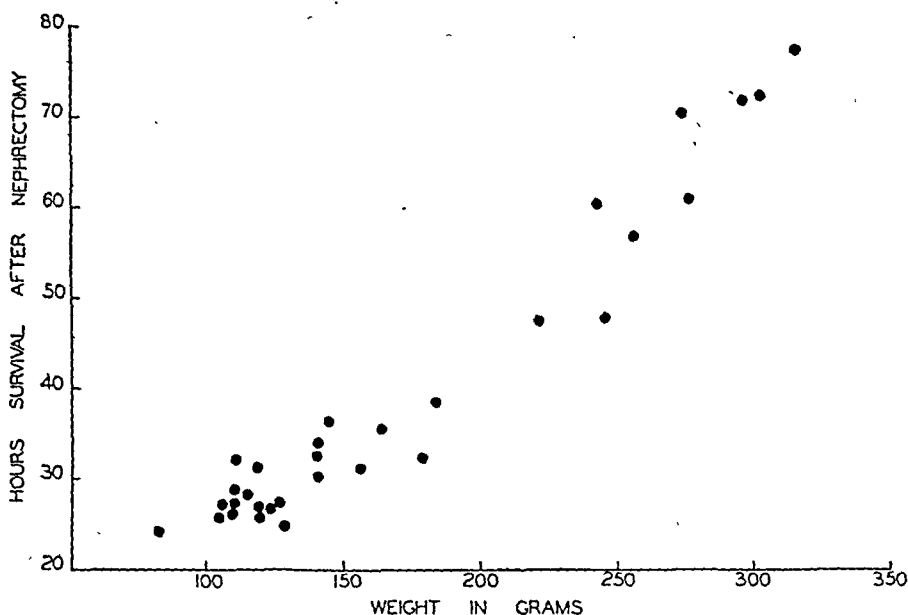
FIG. 1. Showing the relation of body weight to survival period in untreated nephrectomized rats. Each dot represents an individual animal. It is seen that large animals survive much longer than small ones.

ceiving water survived longer than 5 hours from the time the first dose of water was given, whereas 20 of the 24 DCA-treated animals survived more than 5 hours. Survival in the latter group ranged up to 18 hours, but since the exact time of death was not observed in all cases, those living more than 5 hours are grouped together in Figure 2.

The secondary signs of water intoxication, such as collapse, convulsions and tonelessness, but with the exception of body temperature changes noted below, appeared earlier and more uniformly in the oil-treated animals than in the DCA-treated cases. The convulsions of the oil-treated animals, although occurring early and frequently, were of the mild sort characteristic of adrenalectomized animals (Gaunt, 1944), while the less frequent ones of the DCA-treated animals were of the more violent type characterizing the water intoxication in intact animals.

Preliminary experiments indicated that this protective action could not be consistently demonstrated when a bilateral adrenalectomy-nephrectomy was performed at one sitting; the results were dependent upon a two-stage operation.

The body temperature changes constituted an unexpected exception to the rule that the usual criteria of water intoxication were prevented by DCA. Rowntree (1926) first pointed out that a loss of body temperature was characteristic of water intoxication when he described the syndrome. The usefulness of temperature readings as an index of the extent and degree of toxic hydration has been pointed

all groups. Plasma protein levels were reduced somewhat on the average, but as in earlier studies (Gaunt, 1944) the individual results were erratic. A rise in hematocrit and hemoglobin levels occurred in the controls, again as expected from previous work, but this was entirely prevented or even reversed by DCA treatment.

Water intoxication in animals nephrectomized only (Series D: Table 2). Since it was clear that DCA protected adrenalectomized-nephrectomized rats from water intoxication, it became of interest to see if the same effects could be demonstrated in animals from which the kidneys but not the adrenals were removed. It is to be remembered that DCA will protect intact animals against excess water (Gaunt, 1943). As seen in Table 2, clearly negative results were obtained; DCA exerted no detectable beneficial effects after nephrectomy.

Since adrenal cortical extract is more effective in preventing water intoxication in intact animals than DCA, it was tried in nephrectomized animals. The doses of cortical extract used were known to be effective diuretic stimulants in animals with functional kidneys. Like DCA, however, it had no protective action whatever against water intoxication in nephrectomized rats.

Extra amounts of cortical hormones, therefore, do not add to the action of the animal's own adrenals in providing resistance to excess water after nephrectomy. It follows then that the marked effect of these hormones in intact animals as previously reported is due primarily to the fact that they stimulate diuresis. In adrenalectomized animals, an extrarenal action can be demonstrated as well.

DISCUSSION

The results indicate that DCA extends the life-span of adrenalectomized animals longer than will the presence of the animals own adrenals after nephrectomy alone. This is a somewhat anomalous observation in view of the fact that most of the well-known actions of DCA are on the kidney. Its explanation, however, probably lies in the work of Bondy and Engel (1947) who pointed out that by stimulating protein catabolism the natural adrenal secretions may cause in nephrectomized animals a deleterious accumulation of protein metabolites. This protective action of DCA can be demonstrated without pretreatment with the hormone or preoperative dietary manipulation such as have been used by other workers in related experiments (Selye, 1940; Selye and Nielsen, 1941; Bondy and Engel, 1947). The demonstration of this extra-renal action of DCA in extending life apparently depends upon the technique used; in our hands it was not seen after bilateral nephrectomy and adrenalectomy at one sitting but only after a two-stage operation. The discrepancy betweeen thse results and those of Ingle and Kendall (1936) possibly were due to either the operative technique (they used single-stage operations) or differences in the size of animals employed. (See Figure 1.)

The results show clearly that the protective action of DCA against the well-known susceptibility of adrenalectomized animals to excess water (Eversole, Gaunt and Kendall, 1942) is not due entirely to the fact that the hormone facilitates diuresis. Rather, it provides resistance against the toxic action of water as such. Our findings would be consistent with the hypothesis of Hays and Mathieson (1945) that the major action of DCA in this regard is extra-renal.

The mechanism by which DCA counteracts the deleterious effects of hydration is, like the mechanism of water intoxication itself, obscure. One clear effect of DCA was to prevent the rise in hemoglobin and hematocrit levels usually seen in water intoxication, especially in adrenalectomized animals. One could postulate in general terms from this fact that DCA maintains in some way a more favorable distribution of excess water than occurs in its absence; in terms of current theory (Peters, 1944), this presumably would mean that it helps prevent cellular hydration.

The interpretation, however, of a hematocrit rise and a plasma protein fall in the presence of excess water is difficult, especially since conditions are such that dye measurements of fluid compartments may be unreliable (cf. Overman, 1946). The problem has been discussed elsewhere (Gaunt, 1944 and 1946; Shipley, 1945).

The observation that the administration of neither DCA nor adrenal cortical extract had any beneficial effect on water intoxication in nephrectomized animals, in contrast to the helpful action of DCA in adrenalectomized-nephrectomized rats, is not necessarily an anomalous discrepancy. Water intoxication no doubt stimulates adrenal function like any other stress and animals with intact adrenals have a high endogenous output of cortical hormone. Thus the endogenous output is perhaps sufficient to do all that any amount of adrenal steroids can do in making the most advantageous internal distribution of excess water. It follows then that the beneficial effects of cortical hormone in protecting normal animals against water intoxication as previously reported (Gaunt, 1943) is primarily due to a stimulation of the diuretic rate. In adrenalectomized animals extra-renal factors are involved as well.

SUMMARY

In untreated nephrectomized rats the survival period was related to body weight; large animals lived longer than small ones.

Treatment with desoxycorticosterone acetate (DCA) extended the life-span of adrenalectomized-nephrectomized rats even when treatment was started after nephrectomy. Animals given this treatment lived longer than did those from which the kidney but not the adrenals were removed.

A protective action of DCA against water intoxication was demonstrated in adrenalectomized rats, but neither DCA nor adrenal cortical extract increased resistance to excess water in animals from

which the kidneys were removed but the adrenals left intact. The protective actions of cortical hormones against excess water in normal animals is therefore presumably due to stimulation of diuresis, whereas in adrenalectomized animals extra-renal factors are also involved.

In adrenalectomized-nephrectomized animals, forced into water intoxication, treatment with DCA had the following effects: a, the expected rise in hematocrit and hemoglobin was prevented; b, there was a reduction of questionable significance in plasma protein; c, intestinal absorption of water was facilitated slightly; and d, no change in the plasma chloride was seen.

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NOTES AND COMMENTS

ASCORBIC ACID CONCENTRATION AND CHOLESTEROL STORAGE IN THE CORPUS LUTEUM OF THE PREGNANT RAT UNDER EXPERIMENTAL CONDITIONS[1]

CONSIDERABLE interest has lately been attached to the interrelationships between cholesterol metabolism and ascorbic acid in the adrenal cortex. In that organ a sharp fall in ascorbic acid concentration occurs within an hour after injection of adrenocorticotrophin, followed later by marked depression of cholesterol content (Sayers et al., 1946). A recent report from this laboratory (Everett, 1947) presents evidence which indicates that in the corpus luteum of the pregnant rat cholesterol is mobilized in similar fashion by action of the lactogenic hormone (luteotrophin). Although the normal rat corpus luteum of pregnancy contains little cholesterol which can be detected in frozen sections by the Schultz histochemical test, rich intracellular deposits are found 18 hours after injection of an ovulating dose of sheep LH. An excess of lactogen (luteotrophin) however, prevents accumulation of sterol if injected at the same time as LH.

In view of the apparent correlation in the adrenal cortex between ascorbic acid and cholesterol shifts, it appears important to determine whether a similar correlation exists in the corpus luteum. Other investigators (Biskind and Glick, 1936; Pincus and Berkman, 1937) have previously sought a relationship between corpus luteum secretory activity and ascorbic acid, but only by means of assays made at various stages of the estrus cycle and pregnancy. In the rat, for example, Laqueur and Koets (1945) reported that in late pregnancy the total cholesterol content rises while ascorbic acid is considerably diminished.

Our method affords a more direct approach to the question since at a given stage of pregnancy (6 days in our standard procedure) corpus luteum cholesterol content can be modified at will. The specific problems which we explored are as follows: (a) development of a suitable ascorbic acid technique for this particular tissue; (b) determination of ascorbic acid concentration in the normal corpora lutea on the 6th day of pregnancy; (c) ascorbic acid concentration in corpora lutea of similar animals 12 and 18 hours after injection of LH, i.e. shortly before cholesterol storage begins and when the process has reached completion; and (d) the luteal ascorbic acid concentration in animals treated with LH 18 hours previously and injected with an excess of lactogen 1 hour before autopsy.

MATERIALS AND METHODS

Sexually mature albino rats of the Vanderbilt (Osborne-Mendel) strain were used. Pregnancies were accurately timed by controlled mating. Each

·NEW BOOK

TEXTBOOK OF ENDOCRINOLOGY. By Hans Selye xxxii+914 pp. Acta
Endocrinologica Université de Montreal, Montreal, Canada, 1947.

Fifty-nine years ago the venerable Charles Édouard Brown-Sequard
arose before the Societé de Biologie and told a preposterous story to a spell-
bound audience. Aqueous extracts of testicles, self-administered, he said, had
restored the spring to his step and the gleam to his eye. When he had fin-
ished, a new science had been born. True, the new field had been heralded by
the less spectacular (and better founded) observations of Hunter, Berthold,
Bernard, Addison and Gull, but the dramatic account of his subjective ex-
periences captured the imagination of his hearers and made Brown-Sequard
the father of endocrinology. Small wonder that endocrinology. so begun, has
remained a field of fact and fancy, of lurid claims and sober investigation,
and a battleground for the opposing philosophies underlying clinical service
and scientific investigation.

For, make no mistake, a deep chasm separates medical science from
medical practice. Illogical as it is, this separation has been evident at every
stage of medicine's development. Percussion was fought even by the students
of Augenbrugger and Corvisart. Laennec's stethoscope was maintained to be
a contrivance of no value in practice. The same outcry greeted the clinical
thermometer, anesthesia, the blood-pressure cuff and radium. Nor is the
misapprehension confined to the introduction of new methods. It goes much
deeper into the fabric of medical thought and fundamentally condemns
science itself as being in conflict with the humanity which should charac-
terize the physician in the presence of suffering (Flexner, 1925).

Now, such thinking is sophistry. Its inherent fallacy has always been
known to the philosophers of medicine. Beginning with Hippocrates, clinical
medicine has striven to recognize the symptoms which occur in regular com-
binations and sequences and to abstract these combinations from individual
patients into units of classification which are diseases. Such abstraction
brought order into the chaos of symptoms and made possible clinical science,
since classification is the basis of science. Claude Bernard (1865) recognized
clearly that the fundamental reasoning ·of investigators and practicing
physicians is—or should be—exactly the same. Both should attempt to
expel superstition and empiricism by critical observation, reflection, verifica-
tion and generalization. Neither the practitioner nor the investigator should
be blinded by prejudice or jump to conclusions. Both in the clinic and in the
laboratory the severest effort possible should be made in the direction of
purifying, organizing and extending knowledge (Flexner, 1925).

These principles seem clear if not self-evident. Once enunciated, they are
applicable from time to time in different areas of medicine. Yet, it would
seem they must be reasserted for every developing specialty. Dr. Selye's
textbook, intended to " . . . comprise all those facts which lend themselves
particularly well for conjoint study by the same individual" and " . . . to act
as a standard textbook of endocrinology," is far from achieving a balance
reconciling the twin obligations of medicine to treat the sick and to advance

knowledge. It appears, rather, that he regards these two responsibilities as incompatible, for, on page 38, he states:

"I believe that experimental medicine is not an abstract, but an applied science. Admittedly, in the early stages, it is often difficult to foretell whether a problem will have important applications to practical medicine, but at least we should select our problems keeping in mind that practical applicability is their most noble goal. Medical research undertaken merely as a sophisticated type of mental gymnastics—a viewpoint often defended by the slogan 'science for science's own sake'—appears unworthy of the traditions of the medical profession, whose primary aim has always been, and should remain, to help the sick."

This quotation heads up the shortcomings of Dr. Selye's textbook. Although experimental findings are presented, they are arranged and classified according to their utility in recognizing or treating endocrine diseases rather than according to the logical exposition of physiological processes. This organization leads to strange contrasts. For example, the section on the ovary is presented in 147 pages and includes a lengthy classification of ovarian diseases, whereas the sexual cycle, pregnancy and lactation are relegated to subsections in a scrap-basket chapter called Correlations, and altogether comprise only 30 pages. Hepatic hormones are dignified by a headline in the Table of Contents, through which one searches in vain for a reference to the placenta. Thus, everywhere the book over-emphasizes the subjective utility of knowledge and fails to consider that there is an objective endocrinology which exists quite regardless of what the writer may or may not think about it. Annoyed by a similar insistence on practicality, Mall, the anatomist, once wrote to a friend:

"It is time to call a halt on the utility of science. As though it were of no importance to us to understand or to think, unless some old financial tyrant could rob Mother Earth and his fellowman a little more. Or ridding the earth of superstitions, like witchcraft and maternal impressions, were not of far greater value to us than to cure some more sick." (Sabin, 1934)

All this is not to decry the practical usefulness of science. It is merely to emphasize that without objectivity, medicine becomes empirical and is guided only by what is practical. But what works today may not do so tomorrow, and without the utmost effort to construct standards on rational principles, fads will develop and hold sway by the force and authority with which they are advanced.

Once the most rigorous objectivity has been abjured, a specious atmosphere of authority is created and the author is beguiled into ever more dogmatic opinions. To cite a single example, in a section devoid of references, Dr. Seyle writes: "The therapy of choice, in all serious types of hyperthyroidism is the subtotal removal of the thyroid gland." Much has been written pro and con the medical treatment of hyperthyroidism with anti-thyroid drugs. In view of the prevailing controversy, one is at least entitled to ask for evidence, personal or documentary, supporting such an unqualified statement.

It has been emphasized above that investigators and practitioners alike should observe, reflect, verify and generalize. The great textbooks of the past have been based upon one or more of these activities. Osler's textbook of Medicine broke from the traditional and scholastic descriptions of disease and substituted for them the first-hand impressions of a phenomenally acute observer, together with his reflections upon the natural history of disease

and treatment of the patient. This, the present textbook of endocrinology does not do. It is, rather, a hasty and frivolous compilation of unrelated fact or observation passing for fact, written in an authoritative manner with infrequent citations of the sources. Claude Bernard (1865) and William Bayliss (1914) write accounts which are milestones in physiology. Their books represent penetrating searches for the fundamental generalizations of the subject. The discussion above indicates that this was not the aim of the present volume.

There is no question of the desirability of a standard textbook of endocrinology. The literature is enormous, the facts are legion and the methods are specialized. Already, subspecialization is occurring—for example, there is little contact between those who work with the chemistry of steroids and the experimental morphologists. A book in which the basic generalizations were formulated would draw endocrinology together into a coherent whole. Dr. Selye's previous work indicates clearly that he has the qualities of mind required for such a task. His investigations on the adaptation reaction, on the pharmacological classification of steroid hormones and on the methodology of endocrine research exemplify high abstractions in endocrinological thinking. They indicate that he could write a definitive textbook of endocrinology, and it is to be regretted that this is not it.

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ENDOCRINOLOGY

THE EFFECT OF METHYLTHIOURACIL ON THE OXYGEN CONSUMPTION OF THE THYROID, LIVER AND KIDNEYS

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An ABUNDANT literature has made its appearance as a result of the observations that the sulphonamides, thiocarbamides, and thiouracils provoked hyperplasia of the thyroid (Astwood, 1943, 1944; Kennedy, 1942; Mackenzie and Mackenzie, 1943, 1944; Mackenzie, Mackenzie and McCollum, 1941; Richter and Clisby, 1942), and that the basal metabolism falls in proportion as the hyperplasia increases (Astwood, Sullivan, Bissell and Tyslowitz, 1943). Investigations have been chiefly directed on analyzing more in detail the mechanism of the effects of these substances. The hyperplasia of the thyroid proved to be conditioned by an increased excretion of thyrotropin, which in its turn was due to a decrease in the formation of the thyroid hormone, whereby the checking effect of the hormone on the anterior lobe of the hypophysis declined (Astwood and Bissell, 1944; Astwood, Sullivan, Bissell and Tyslowitz, 1943; Higgens and Ingle, 1946).

Various methods have been employed to elucidate how these substances influence the synthesis of the thyroid hormone. In *in vitro* experiments, by adding thiouracil to the substrate, it was possible to show in the thyroid section that the peroxidase reaction of the follicle cells was checked, while the cytochromoxidase reaction was not affected (Dempsy, 1944). This observation would seem to argue in favour of the peroxidase being necessary for the synthesis of thyroxin, and indicate that the effect of the antithyroid substances is a checking of the effect of this enzyme.

However, it is in the first place by the use of radio-active iodine in experiments both *in vivo* and *in vitro* that attempts have been made

to reveal the mode of action of these substances. In the case of *in vivo* experiments, it has been proved that the capacity of the thyroid to take up I is greatly reduced after thiouracil treatment (Franklin, Chikoff, and Lerner, 1944; Keston, Goldsmith, Gordon and Charipper, 1944; Larsson, Kesting, Peacock and Rawson, 1945; Rawson, Tannheimer, and Peacock, 1944). According to others the I-uptake is not checked, but the thyroid cells themselves are attacked, which leads to a decreased synthesis of the thyroid hormone (Baumann, Metzger and Marine, 1944). The amounts of diiodotyrosine and thyroxin decrease after medication with antithyroideal substances (Franklin, Chaikoff and Lerner, 1944). In *in vitro* experiments the storage of I was not checked, but its entry into diiodotyrosine and thyroxin was checked (Franklin, Chaikoff, and Lerner, 1944; Keston, Goldsmith, Gordon and Charipper, 1944; Lerner and Chaikoff, 1945). In any case these experiments seem to have shown that the synthesis of the thyroid hormone is seriously interfered with. On the other hand, opinions appear to differ as to whether there is a disturbance in some enzyme system, or whether it is a question of an injury to the follicle cell itself.

With the help of the Warburg method, attempts have been made to analyse which enzyme system or systems are checked. Thus, in *in vitro* experiments, thiouracil in a 0.002 M solution is said to check cytochromoxidase (Paschkis, Cantarow, Rakoff and Tillson, 1945). On the other hand, others consider that this enzyme system is not affected (Lerner, and Chaikoff, 1945; McShan, Meyer and Johanson, 1946).

Jandorf and Williams (1944) showed that the oxygen consumption of the thyroid increases greatly in animals which have been treated with thiouracil for 10–20 days. On the other hand, thiourea added *in vitro* does not have any effect on the oxygen consumption of the thyroid section. The different results as between *in vivo* and *in vitro* experiments are explained by the fact that the cell volume is considerably larger in the former experiments and conditions the increased consumption of oxygen (Lerner and Chaikoff, 1945).

Certain clinical observations, such as blood changes, exanthema, "drug fever," etc. suggest that the antithyroid substances have not a selective effect on the thyroid. Such toxic reactions have also been observed in animals (Mackenzie and Mackenzie, 1943; Meyer, Collins and Marine, 1944). There is experimental support for the suggestion that antithyroideal substances also affect other organs than the thyroid. Thus *in vitro* experiments with thiouracil in 100 mg.% concentration the oxygen uptake of rabbit bone-marrow is checked (Warren, 1945), while, on the other hand, the oxygen consumption of liver from animals which had been treated for longer or shorter periods with thiouracil, was not changed (Jandorf and Williams, 1944).

A considerable period (20 days) of administration of thiouracil in

the food reduced the activity in homogenized liver of both succin-oxidase and cytochromoxidase. The cause of this was the reduced syn-thesis of the thyroid hormone. If thyrotropic hormone, dried thyroid, or thyroxin are administered for 2 weeks a definite increase in the activity of the liver enzyme results. In experiments with liver from both thyroidectomized and thiouracil-treated rats the same activity in the enzyme system in question resulted in both cases, which would suggest that thiouracil does not affect organs other than the thyroid (Tripton and Nixon, 1946). The question whether the antithyroid substances only attack certain enzyme systems in the thyroid which are necessary for the synthesis of thyroxin, or whether they have a more general point of attack is thus still uncertain.

Only one work has dealt with the changes in the oxygen consump-tion of the thyroid after treatment with thiouracil (Jandorf and Williams, 1944), and the conclusions are based on very few experi-ments. It therefore appeared to us of interest to carry out such an investigation on a large animal material, and further to attempt to show whether thiouracil has a more general point of attack or only affects the tissue of the thyroid.

MATERIAL AND METHODS

For the present work white rats, all males, were employed. The animals all received the same food. The drinking water consisted of a 0.1% methyl-thiouracil solution, to which the animals had free and abundant access, and which was renewed every day. The animals were killed after having been on the diet and drinking water indicated here from 1 to 67 days (see table 1). Immediately after the animal had been decapitated a piece of liver, kidney, and left thyroid lobe were excised for determinations of the QO_2 according to Warburg, With regard to the method, see Borell (1945); Holmgren and Naumann (1947).

In some cases we determined the oxygen consumption of liver and kidney from thyroidectomized rats. The animals were given methylthiouracil in the drinking water about 10 days after the operation, when they had recovered from the operation.

The right lobe of the thyroid was always fixed in Susa for 24 hours and subsequently embedded in paraffin in the usual manner.

From three symetrically chosen heights within the lobe of the gland sections 5μ thick were taken and stained with hematoxylin-eosin.

RESULTS

As appears from table 1 and diagram 1, the oxygen consumption for the thyroid is relatively high in the control animals—QO_2 of —7.6. In all probability this is due to the fact that normally the thyroid of rats is active, with high follicle epithelium and but little colloid. Thyroid from guinea-pigs of the same size give QO_2 values of only —3.7 (Borell, 1945) which tallies well with the inactive picture shown throughout by these glands. Even these figures show that

glandular activity and QO_2 are interdependent, a circumstance that appears clearly from Borell's work on cell height and oxygen consumption in cases of treatment with increasing doses of thyrotropine.
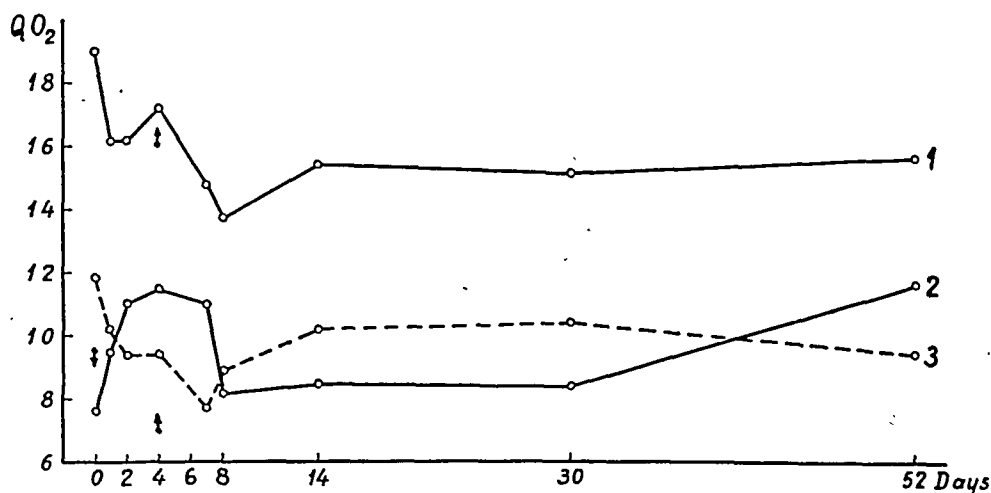
DIAGRAM 1

The oxygen consumption of thyroid, liver and kidney.
    1 = Kidney
    2 = Thyroid
    3 = Liver
    ♂ = Liver and kidney from thryoidectomized animals and treated with methyl-
        thiouracil four days.
    ♀ = Liver from only thyroidectomized animals.

TABLE 1. THE QO_2 IN THE THYROID, LIVER AND KIDNEY FROM RATS
TREATED WITH METHYLTHIOURACIL

Number of days treated with methylthiouracil	QO_2		
	Thyroid	Liver	Kidney
Controls	$- 7.6\ n=10$	$-11.8\ n=22$	$-19.0\ n=9$
1 day	$- 9.5\ n=7$	$-10.2\ n-7$	$-16.1\ n=6$
2 days	$-11.0\ n=6$	$- 9.4\ n=6$	$-16.2\ n=6$
4 days	$-11.4\ n=8$	$- 9.4\ n=6$	$-17.2\ n=8$
7 days	$-11.0\ n=6$	$- 7.7\ n=6$	$-14.8\ n=6$
8 days	$- 8.1\ n=6$	$- 8.9\ n=6$	$-13.7\ n=6$
14 days	$- 8.5\ n=8$	$-10.2\ n=8$	$-15.4\ n=5$
30 days	$- 8.4\ n=6$	$-10.5\ n=5$	$-15.1\ n=4$
67 days	$-11.7\ n=12$	$- 9.4\ n=12.$	$-15.7\ n=12$

Even in animals which had drunk water containing methylthiouracil for only 24 hours the QO_2 in the thyroid increased to .— 9.5. The difference between this value and that exhibited by the control animals is not significant; the great dispersion within the respective groups is remarkable. In the case of the rats which received methylthiouracil the cause must be that the dosage naturally varied from animal to animal, all depending on the amount they drank during the first day of the experiment. Within the control group also the disper-

sion is great, which is explained by the fact that the activity picture of the thyroid in rats varies greatly from case to case.

In rats which received methylthiouracil for 2 days there is a further increase in the QO_2 to -11.0. The difference from the value for the control animals is now statistically probable, with a t-value of 2.86 and $p < 0.02$. On the other hand, the difference between the control animals and the animals which had received methylthiouracil for 4 days is significant. As appears from table 1, the QO_2 value is about -11 for animals which had consumed thiouracil for 4 to 7 days, but after 7 days a very marked fall in the QO_2 takes place down to values of -8. The fall is statistically verified, with a t-value of 3.30 and $p < 0.01$ (see table 2). Finally, we found the highest QO_2 value for the oxygen consumption of the thyroid from rats which had received

TABLE 2. STATISTICAL CALCULATIONS OF THE DIFFERENCE BETWEEN THE MEAN VALUES FOR THE QO_2 IN THE THYROID, LIVER AND KIDNEY

Groups between which the difference was investigated		t	p	Organ
A.	B.			
Normal animals	1-day animals	1,84	<0,1	
Normal animals	2-day animals	2,86	<0,02	
Normal animals	4-day animals	4,01	<0,01	Thyroid
4-day animals	8-day animals	3,30	<0,01	
30-day animals	67-day animals	2,70	<0,02	
Normal animals	1-day animals	2,08	<0,05	
Normal animals	2-day animals	3,42	<0,01	
Normal animals	4-day animals	3,40	<0,01	
7-day animals	8-day animals	1,25	<0,3	Liver
7-day animals	14-day animals	2,17	<0,1	
7-day animals	30-day animals	3,06	<0,02	
30-day animals	67-day animals	0,66	<0,6	
Normal animals	1-day animals	1,59	<0,2	
Normal animals	2-day animals	2,00	<0,1	
Notmal animals	4-day animals	1,18	<0,3	
Normal animals	7-day animals	4,53	<0,001	
Normal animals	8-day animals	3,94	<0,01	Kidney
8-day animals	67-day animals	0,84	<0,5	

methylthiouracil for 67 days. The difference (-3.3) between this value and the value for the 30-day animals (8.4) is statistically probable with $p < 0.03$.

It is of special interest that the liver and kidney respiration changes during methylthiouracil medication in quite a different manner from that of the thyroid. As appears from table 1 and diagram 1, for these two organs the QO_2 value falls already during the first 24 hours of the treatment, and the reduction continues, successively on the whole, until minimum values are reached after 7–8 days. The liver respiration of the control animals (-11.8), which tallies well with other observations (Holmgren and Naumann, 1947), differs significantly from the QO_2 for liver from rats killed after 2 days' treatment

(-9.4). The difference is -2.4 and the t-value 3.42, with a $p < 0.01$.

On the other hand, a statistically verified difference in kidney respiration as between the control animals and those treated with methylthiouracil does not show itself until after 7 days' treatment, with a t-value of 4.53 and $p < 0.001$. If the treatment is continued for a considerable period, 30 to 67 days, the QO_2 for liver and kidney shows no further change.

It is obvious that the kidney sections within the respective groups give very varying values for QO_2, and the dispersion is remarkably great.

A group of 12 rats were thyroidectomized, and 5 of them were put on water containing methylthiouracil about 10 days after the operation and kept on this fluid for 4 days, after which they were killed, and the oxygen consumption determined on liver and kidney sections. These experiments were made to ascertain whether methylthiouracil possibly affected other organs than the thyroid. The QO_2 in rats which were only thyroidectomized was -9.2, which is less than that of the normal animals. The QO_2 in the liver of rats which had been operated on and received methylthiouracil was considerably lower, however, amounting to -6.9. The difference between the liver respiration in the respective groups is statistically probable, with t-values of 3.11 and $p < 0.02$. The corresponding values for kidney sections were -16.0 and -16.3 respectively. The individual values in the different experiments appear from table 3.

DISCUSSION

A more detailed analysis of the results of the experiments renders possible a discussion of the mode of action of methylthiouracil.

The actual synthesis of thyroxin is associated with oxidative processes, chiefly in the conversion of iodide to free iodine. However, according to a number of investigations with radioactive iodine, methylthiouracil appears to check just that process.

Thus the increase which we found in the oxygen consumption of the thyroid gland may not be due to an increased formation of the hormones of the gland. Just by investigating thyroids treated with methylthiouracil a possibility is presented of a closer study of the changes conditioned by processes in the gland other than those directly associated with hormone function.

The circumstance that there is an increase in the oxygen consumption in the thyroid, but a reduction in the consumption of the liver and kidney, does not exclude the possibility that it is the same enzyme system in the different organs that is affected, for there is much which indicates that the increase in the oxygen consumption of the thyroid after methylthiouracil is chiefly conditioned by an increase in the size and number of the follicle cells of the thyroid. If the QO_2 is calculated, and not the total oxygen consumption of

the gland, the effect of an increased volume of the gland is avoided. It is then probably only the cell increase itself which is registered. Experiments which have been carried out earlier also speak in favor of the correctness of this argument, for,· in the case of injections of small amounts of thyrotropin, a great rise in QO_2 was met with during the time the follicle cells increased greatly in size, but, in spite of further injections over a couple of days, no changes could be proved either in cell height or QO_2 (Borell, 1945). Other experiments also ·indicate the correctness of this. By determining the number of mitoses in the thyroid after treatment with methylthiouracil for varying numbers of days, it has been possible to show that on the whole the changes in the number of mitoses follow the changes we found in the QO_2 (Paschkis, Cantarow, Rakoff and Rothenberg, 1945; Rerabek, 1946). That the increase in the QO_2 of the thyroid is not a direct effect of the methylthiouracil appears from the fact that the QO_2 in the thyroid decreases, in spite of continued supplies of methylthiouracil.

It appears to be of interest that the histological picture of the thyroid is still active.after more than 60 days' methylthiouracil treatment, for, according to a number of authors, repeated injections of thyrotropin provoke a return to inactivity, owing to the formation of an antithyrotrophic substance (Anderson and Collip, 1934; Collip and Anderson, 1935; Eitel and Loeser, 1935). Others, however, state that the cause is the development of antibodies against the unspecific protein substances which accompany the preparation extracted from the anterior lobe (Katzman, Wade and Doisy, 1937; Werner, 1936). Our experiments support this opinion, as in the rats treated with methylthiouracil the content of thyrotropin increases and maintains a histological active thyroid gland. In these cases naturally no unspecific protein is met with.

If the synthesis of the thyroid hormone is entirely stopped by methylthiouracil, it should be expected that, on the whole, the oxygen consumption of the liver would be the same in thyroidectomized animals as in those treated with methylthiouracil. The results show that, on the whole, the same values are obtained under both sets of experimental conditions. It is possible that the oxygen consumption is somewhat higher in the treated animals. An explanation of this may be that the synthesis of thyroid hormone is not entirely abolished. Further, it is known that the effects of thyroxin do not disappear rapidly but persist for a very long time. Thus it should still be possible to trace the effect after about a month. Others have found no change in the oxygen consumption of the liver after methylthiouracil (Jandorf and Williams, 1954). Thuss we cannot verify these results. The reason must be that those authors used small series, and their values shows great dispersion.

Results of the experiments in which thyroidectomized animals were treated with methylthiouracil also appear to be of interest (table

3). In this case the respiration of the liver was considerably lower than that of the control animals, which had only been thyroidectomized. This definitely indicates that methylthiouracil also attacks other organs than the thyroid. In similar experiments certain enzyme systems taking part in the oxidative processes have been investigated, but no difference could be proved as between the experimental series (Tripton and Nixon, 1946). To a certain extent this conflicts with our results, but it may be explained by the fact that these authors only determined the content of individual enzyme systems, while in our experiments we measured the total consumption of oxygen.

TABLE 3. THE QO₂ IN KIDNEY AND LIVER SECTIONS FROM THYROIDECTOMIZED ANIMALS

Liver		Kidney	
Controls	Treated with methylthiouracil	Controls	Treated with methylthiouracil
10,2	5,6	11,1	13,9
7,4	6,9	16,8	19,2
11,1	7,1	18,5	15,9
7,2	7,9	14,4	16,0
9,8	7,1	15,7	16,3
10,2		19,7	
8,8		15,9	
9,24	6,92	16,01	16,26

Finally, with regard to the respiration experiments with the kidney, on the whole the QO_2 in the kidney changed analogously with that of the liver. Since the dispersion of the individual values was very great, statistically verified differences from normal values were not obtained until after 7 days' treatment with methylthiouracil. After thyroidectomy the same oxygen consumption in the kidney is found, both with and without methylthiouracil. The individual values exhibit very great dispersion (table 3). The cause of this is probably that tangential sections of the kidneys were taken, in which case the quantity of medulla and cortex varies greatly from section to section. These two parts of the kidney have different QO_2 values, and therefore the variations in their amounts probably play an important role.

SUMMARY

In the present work the effect of methylthiouracil on the consumption of oxygen in the thyroid, liver and kidney, has been studied. Further, the effect of this substance on thyroidectomized animals has been investigated. On the whole the results were as follows:

The oxygen consumption of the thyroid increases rapidly after the administration of this substance in the rats' drinking-water. The increased consumption is probably due to growth of the follicle cells of the thyroid. In spite of further supplies, the respiration falls.

The livers from thyroidectomized animals which have been treated with methylthiouracil show a lower oxygen consumption than do those of the control animals. This argues definitely in favor of the assumption that the methylthiouracil had a general effect and did not selectively attack the thyroid.

The oxygen consumption of the liver and kidney falls relatively soon after methylthiouracil treatment is begun. The cause of this appears to be a decreased synthesis of thyroid hormone. Further, there is a clear—at least as far as the liver is concerned—direct effect of methylthiouracil.

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HYPERVOLEMIA IN MICE BEARING GRANULOSA CELL GROWTHS; TIME OF ONSET AND SOME ASSOCIATED PHYSIOLOGICAL AND CHEMICAL CHANGES[1]

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IT WAS NOTED recently that mice bearing subcutaneous growths of transplanted granulosa cells have enormously congested livers and adrenals (Furth and Boon, 1945) and an associated hypervolemia with 2 to 3 times normal values (Furth and Sobel, 1947). The hypervolemia is present in mice bearing transplanted granulosa cell tumors originally induced by x-rays (Furth and Sobel, 1946) or produced by intrasplenic grafts of ovarian fragments into gonadectomized mice (Furth and Sobel, 1947). It was suggested that this increase in blood volume was produced, directly or indirectly, by a hypothetical substance elaborated by the neoplastic granulosa cell. The physiological and chemical studies reported here were undertaken to learn more about the nature of this hypervolemia.

MATERIALS AND METHODS

The origin of the transplantable granulosa cell tumors used in this experiment has already been described (Furth and Sobel, 1946; 1947). Strain V, an x-ray induced tumor, was in its 8th and 9th passage with one exception: the tumor carried by mouse 1483 was in its 3rd passage and the similar strain B2 in its 3rd and 4th passage. A few tumors of Strains I and III were also studied. The tumors examined have been growing faster than in the earlier passages and this may account for the decreased degree of associated hypervolemia.

In order to determine the blood volume at repeated intervals the technic previously used (Furth and Sobel, 1946) has been modified so that samples could be taken without killing the mouse. 0.20 cc. of Evans Blue solution (0.15–0.30 per cent in saline) was injected into a tail vein of a mouse that had been anesthetised with nembutal. The jugular vein was exposed through a small incision and 0.1 or 0.2 cc. of blood was drawn into a small syringe, that had been rinsed with heparin solution, using a 27 gauge needle. Bleeding was stopped by pressure and the wound was sutured.

The repeated determinations could be carried out one week apart for it was found that the dye was removed from the blood stream by this time. This procedure has been repeated up to 5 times on the same mouse. It was important to reduce trauma and infection to a minimum since scarring made subsequent bleedings more difficult.

To 0.10 cc. of the blood sample was added 0.90 cc. of isotonic sodium citrate solution. After centrifugation the concentration of the dye in the supernatant was determined at 575 mμ in the Beckman spectrophotometer[3] using microcells with approximately 0.7 cc. capacity described by Lowry and Bessey (1946).[4]

When the thiocyanate space was measured simultaneously with blood volume (Gregersen and Stewart, 1939), 1.0, 1.5 or 2.0 mg. of sodium thiocyanate in 0.2 cc. of solution was injected into the tail vein of mice 24 minutes before the injection of the dye. The mouse was exsanguinated. 0.1 cc. of plasma was put into 0.65 cc. of distilled water and the proteins were precipitated by 0.25 cc. of 20 per cent trichloracetic acid. To 0.5 cc. of the supernatant was added 0.5 cc. of the ferric nitrate solution and the color was read at 460 mμ in micro cells of the Beckman spectrophotometer. The difference between the thiocyanate space and plasma volume will be referred to as the "extracellular-extravascular space." The total volume of the solutions injected may have a significant influence on the body water of the mouse and the values obtained are regarded as relative.

The total plasma protein and albumin were determined by the method of Greenberg (1929) using the usual constants but smaller quantities of plasma[5] (0.100 cc. for albumin and 0.050 cc. for total protein). The blood urea was determined by the method of Archibald (1944) using 0.10 cc. of blood.

The water tolerance test was performed by administering by stomach tube water equivalent to 5 or 10 per cent of the body weight. When 10 per cent was given, it was administered in two doses at 30 minute intervals. The feces and urine were first expelled by gentle massage and the mouse was placed in a metabolism cage made by suspending a wire support into a 4 inch funnel sealed at the end of the calibrated stem. The urine volume was measured at hourly intervals for 3 hours. The results were expressed as the ratio of the per cent of water recovered after the third hour from the tumor bearing mouse to the per cent recovered from the normal control mouse.

For blood glucose determination by a modification of the Folin-Wu method (1920) 0.02 cc. of blood was obtained from the tail of the heparinized mouse using a special white cell counting pipette. The blood was diluted in the pipette, transferred to 0.70 cc. of water and the proteins were precipitated with 0.10 cc. of tungstic acid. To 0.70 cc. of the supernatant fluid 0.50 cc. of the copper reagent was added and the air was excluded by a glass plug. After the usual heating period the cooled mixture was treated with 2 cc. of a 1:1 mixture of phosphomolybdic acid reagent in glacial acetic acid and the concentration was determined using a visual colorimeter with micro attachments.

When glucose tolerance was determined 0.004 cc. per gm. of mouse of a

TABLE 1. REPEATED BLOOD VOLUME DETERMINATIONS ON MICE BEARING GRANULOSA CELL TUMORS

Mouse No.	Strain	Tumor size[1]	Days after inoculation	Weight of mouse	Blood vol. % body weight[2]	Red cells	
						$\times 10^5$ per cu. mm.	$\times 10^8$ per gm.[3]
2134	V	18.3	55	26	10.6	8.2	8.7
		20.3	62	30	9.4	9.0	8.5
		21.6	70	34	16.7	10.6	7.7
2189	V	16.3	30	25	9.6	9.3	8.9
		25.0	40	29	18.4	5.2	9.6
2190	V	14.6	38	24	8.7	9.8	8.5
		20.0	48	25	13.3	6.7	8.8
		20.9	55	26	20.9	6.2	12.9
		21.6	62	26	28.4	3.6	10.2
2191	V	15.0	25	21	9.6	7.2	6.8
		19.0	35	20	21.7	4.0	8.6
2207	V	13.6	55	24	10.4	11.0	11.1
		21.3	63	27	9.9	8.6	8.5
		29.2	77	30	18.5	4.0	7.4
2201	V	26.3	47	28	14.1	7.2	10.1
		33.3	52	26.5	18.1	—	—
2348	V	11.6	153	30	9.5	9.5	9.0
		11.6	160	30	10.6	9.7	10.3
		17.3	174	31	10.6	9.2	9.8
		20.2	184	33	11.2	10.1	11.3
		23.0	198	29.5	17.3	7.2	12.4
2383	V	25.3	60	29.5	15.0	10.4	15.5
		27.3	68	28	15.5	8.9	13.7
2375	V	30.6	60	30	14.0	7.1	10.0
		30.6	68	32	20.0	6.0	12.0
1483	V	11.3	389	34	8.1	9.4	7.6
		13.0	447	36	9.2	9.0	8.3
		14.3	466	35	9.8	7.9	7.8
2135	B1	11.3	63	21	10.9	9.1	9.9
		18.5	87	24	11.0	11.2	12.3
		23.0	93	23	9.4	9.9	9.3
		27.6	103	23	17.1	6.3	9.3
2187	B1	14.6	53	24	8.4	12.1	10.2
		25.6	68	27	23.0	5.6	12.9
2188	B1	10.6	77	21	9.0	10.1	9.0
		13.3	85	23	9.3	11.6	10.8
		20.3	92	24	8.9	9.2	8.0
		24.3	102	26	15.6	4.4	6.8
2345	B2	19.6	72	36	9.2	10.3	10.1
		21.3	79	35	10.2	8.4	8.6
		21.3	86	34	11.4	8.3	9.4
		22.9	93	31.5	12.2	7.3	8.8
2389	B2	10.0	115	29	8.8	9.2	8.0
		11.6	136	29	9.5	9.3	8.0
		21.3	189	31	14.3	7.5	10.7
		23.6	199	32	14.7	5.8	8.3
10 mice, normal					8.6–10.9 (9.9)	7.5–10.4 (8.7)	6.4–11.4 (8.6)

[1] The size of the tumor is arbitrarily given as the average of the three greatest diameters as determined by approximate external measurement.
[2] Based on total weight of mouse.
[3] Blood Volume (cu. mm.) $\times$ R.B.C. in millions

$$\frac{}{\text{Weight of mouse}}$$

50 per cent glucose solution was injected into the tail vein. Samples of blood were taken before injection and at 15, 45, 75 and 105 minutes after injection. It was usually necessary to inject heparin again before the 4th specimen was taken.

RESULTS

Repeated blood volume determinations. The data presented in Table 1 show that the time of onset of hypervolemia was unpredictable and that the blood volume rose rapidly once the mechanism was set in motion. E.g. in mouse 2191a hypervolemia was present on the 35th day after inoculation with tumor particles while with mouse 2348 carrying the same strain of tumor hypervolemia did not occur until the 198th day. The blood volumes of mice 2134, 2189, 2191, 2135 and 2188 rose from normal values (below 10 per cent of body weight) to values between 15.6% and 21.7% of body weight in 8 to 10 days.

A correlation of blood volume with tumor size as expressed by the average of the three greatest dimensions revealed that there was a minimal tumor size below which hypervolemia did not appear, and that a mouse may have a very large blood volume while carrying a smaller tumor than another with a normal blood volume. E.g. mouse 2191 bearing a tumor of 19.0 mm. had a blood volume of 21.7% of body weight while mouse 2135 had a tumor of 23.0 mm. with a normal blood volume. Once the blood volume began to rise small increases in tumor size were accompanied by great increases in blood volume. E.g. in mouse 2134 the blood volume increased from 9.4% to 16.7% while the tumor grew from 20.3 mm. to 21.6 mm. The blood volume of mouse 2375 increased from 14.0% to 20.0% while the tumor size was not significantly altered. Successive determinations of blood volume of mouse 2190 yielded figures of 8.7%, 13.3%, 20.9% and 28.4% body weight while the tumor size was 14.6 mm., 20.0 mm., 20.9 mm. and 21.6 mm. respectively. Thus it is evident that the blood volume increases rapidly once it begins to rise.

Strain B2 granulosa cell tumors were associated with only slight hypervolemia, strains B1 and V with marked hypervolemia. Cells of all 3 strains secrete estrogens. (Furth and Sobel, 1946, 1947).

Red Blood Count During Hypervolemia: It was reported previously (Furth and Sobel, 1946) that in hypervolemic animals bearing strain V granulosa cell tumors the total number of circulating erythrocytes had increased although the hematocrit values were usually decreased. Mice bearing the tumors had an average of 10.2×10^8 erythrocytes per gram of mouse while normal mice had an average of 5.3×10^8 erythrocytes per gram.[6]

.This change appears to have been lost in the course of successive passages of Strain V. In the course of the current studies, the total number of erythrocytes per gram of animal was in the normal range

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[6] These values were based on the blood volume as determined by exsanguination—perfusion and are approximately one half of those obtained by the dye technique.

except in mouse 2383 that had a somewhat increased number of red cells. Two mice bearing the B1 tumor (not reported in the table) had 18.2×10^8 and 13.0×10^8 erythrocytes per gram of mouse.

The Extracellular Fluid: The finding of hypervolemia raised the question of the distribution of water in other compartments. By the simultaneous determination of thiocyanate space, blood volume, and hematocrit, the "extracellular-extravascular" space was determined. The fluid in this compartment was appreciably increased in only 3 of

TABLE 2. THIOCYANATE SPACE, BLOOD VOLUME AND "EXTRACELLULAR-EXTRAVASCULAR" FLUID IN MICE BEARING GRANULOSA CELL TUMORS

Mouse No.	Strain	Thiocyanate space % body weight	Blood volume % body weight	"Extracellular-extravascular" fluid % body weight
2343	B2	35.4	12.2	26.8
2326	B2	41.4	12.3	34.5
2409[1]	B2[4]	35.3	12.7	27.4
2348	V	44.3	17.3	35.8
2383	V	41.5	15.5	31.6
2375	V	40.3	20.0	24.7
2491	I	35.3	13.6	26.3
2521	B2[4]	37.5	15.5	27.8
2389	B2	37.5	14.7	27.1
2515	V	37.5	18.4	24.7
Normal Values		28.1–33.5[2]	8.4–10.5[3]	23.5–27.0[3]

[1] Ovarectomized.
[2] 15 determinations.
[3] 12 determinations.
[4] These two mice had splenic grafts of granulosa tumors; all others subcutaneous grafts.

The following determinations were thought to be erroneous and were not included in the table: One "normal" mouse had a thiocyanate space of 37.2% and an "extracellular-extravascular space" of 32.3%. One apparently dehydrated "normal" mouse had a thiocyanate space of 24.5%, a blood volume of 7.7%, and an "extraceullular-extravascular" space of 20.1%.

the 10 mice bearing granulosa cell tumors (Table 2). Mouse 2326 had an "extracellular-extravascular" space of 34.5% while its blood volume was only slightly elevated. On the other hand mouse 2491 had a normal "extracellular-extravascular" space with a blood volume of 20.0% of its body weight. Thus an increase in "extracellular-extravascular" space can occur in mice with granulosa tumors but it does not appear to be primarily related to the hypervolemic process. It is known that estrogens can cause the retention of water (Thorn, Nelson and Thorn, 1938).

Plasma Proteins. The great increase in plasma volume raised the question of its effect on blood constituents. The finding on plasma proteins are presented in Table 3. Of a total of 15 mice bearing granulosa tumors, 4 had subnormal albumin values and one, (2364), had an increased value, the rest were approximately normal. The globulin values were approximately normal in 8 mice, markedly below normal

in 5. Five mice had normal total protein levels. In mice 1414, 1850 and 2146 the exsanguination blood volume was 5 cc. or more, an enormous increase; nevertheless, the albumin levels were normal.

Thus, it is apparent that in nearly all cases this hypervolemia is associated with an increase in the absolute amount of circulating albumin, sufficient to maintain a normal concentration. While there

TABLE 3. PLASMA PROTEIN VALUES OF MICE BEARING
GRANULOSA CELL TUMORS

Mouse No.	Strain	Total protein	Albumin	Globulin	Blood obtained[1] cc.	Congestion[2]	
						Liver	Adrenal
1414	V	4.7	3.7	1.0	6.5	+++	++
1853	V	4.2	3.4	0.8	3.4	++	+
1857	V	4.8	3.6	1.2	4.1	++	+
1925	V	3.2	2.6	0.6	2.0	++	+
1850	V	3.6	3.3	0.3	5.0	+++	++
2105	V	5.0	3.5	1.5	4.8	+	
2153	V	4.9	3.9	1.0	3.0	+++	++
2146	V	3.8	3.3	0.5	5.1	+++	+
2133	B1	4.07	3.29	0.78	1.4	+	
2291	B1	4.63	3.48	1.15	2.8	++	
2132	B1	3.98	3.25	0.73	3.8	+++	
2364	V1	5.33	4.16	1.17	3.7	+++	
2209	B2[3]	4.23	3.14	1.09	1.3	++	
2212	B2[3]	4.12	3.21	0.91	0.7		
2328	B2[3]	4.17	2.89	1.18	0.9		
Average		4.3	3.4	0.9			
Normal plasma[4]							
Extremes		4.4–5.0	3.2–3.7	0.9–1.3	About 1 cc.		
Average		4.7	3.5	1.2			

[1] By cutting jugular vein. These values may be regarded as a crude index of blood volume.
[2] As seen in sections. + =slight; + + =moderate; + + + =advanced congestion.
[3] These tumors were intrasplenic; all others subcutaneous.
[4] Six determinations on plasma from groups of mice, pooled.

was an increase in the total amount of circulating plasma globulins in several mice, this tendency is not as general as with the plasma albumin.

Blood Urea. It was observed that several mice bearing large subcutaneous granulosa cell tumors had oliguria. Furthermore, it was thought that hypervolemia might possibly result from an imbalance of the renal vasoexcitor and hepatic vasodepressor (Schorr, Zweifach and Furchgott, 1945); therefore determinations of blood urea were made. Table 4 shows values obtained on mice with various degrees and at various stages of hypervolemia and that normal, slightly or greatly elevated levels of blood urea accompanied the hypervolemia. This could mean that the increase in blood urea may be a direct or indirect consequence of the hypervolemic process or occurs independently.

Histology of Hypervolemia: Earlier studies have indicated that hypervolemia is regularly associated with cavernous congestion of

several organs, most consistently the liver and (or) the adrenals (Furth and Sobel, 1946). The degree of cavernous congestion in these organs parallels the degree of hypervolemia and, in absence of blood volume determinations, this can be used as a crude indication of its magnitude. However, the relative degree of congestive changes in these organs varies so greatly as to warrant the assumption that vasodilation in one of these organs alone (e.g., liver or adrenal) is not the prime cause of hypervolemia.

Heart Weight. Blood pressure determinations have not yet been made on mice bearing a granulosa tumor. In order to obtain preliminary information as to a possible coexisting hypertension the hearts of many mice with marked hypervolemia were weighed. There seems to be no clear cut relationship between heart weight and congestive changes as indicated by the following figures:

HEART WEIGHTS OF GRANULOSA CELL TUMOR BEARING MICE
Congestive changes

0	+	++	+++
.52*	.40	.39	.48
	.46	.40	.48
	.60	.49	.56
	.57	.44	.66
		.43	
		.46	
		.43	
Average .52	.51	.44	.55

* Percent of body weight.

The normal heart weight of 10 mice varied between .43 and .54 and averaged .48% of body weight.

The heart weights of some animals were above the average of normal while those of others were below normal. These findings are difficult to explain. Since it requires time for the development of changes in heart size following a rise or drop in blood pressure, these findings do not exclude with certainty hyper- or hypotension in the experimental animals. Determinations of blood pressure in mice before the onset of hypervolemia and during its various phases may yield valuable information as to the nature of hypervolemia.

Further preliminary findings with respect to liver and adrenal function. An investigation was begun on the function of the liver and adrenals in mice bearing granulosa cell tumors. The following observations have been made thus far:

(1) Prothrombin time studies on 9 mice bearing such tumors and with obvious hypervolemia yielded decreased, normal, or increased values.[7] The prothrombin values of 11 normal mice determined by the Herbert two stage method varied between 94% and 106.5%

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[7] We are indebted to Dr. W. K. Rieben for these determinations.

averaging 100.18%. Those of 10 mice with granulosa tumors varied between 70% and 124.5%. Two of these mice with subnormal values had liver damage. One had a prothrombin value of 79.5% and a + + congestion in liver associated with a marked leukemoid reaction; another with a prothrombin value of 70% and an advanced cavernous congested liver (+ + +) indicative of a marked hypervolemia and numerous infarcts in the liver. One mouse with a greatly elevated prothrombin value (121%) had a marked liver congestion; another

TABLE 4. BLOOD UREA NITROGEN OF MICE BEARING
GRANULOSA CELL TUMORS

Mouse No.	Strain	Blood urea nitrogen mg. %	Blood obtained by exsanguination
2120	V	98	3.8 cc.
2123	V	88	1.7 cc.
2121	V	26	4.1 cc.
2119	V	112	3.9 cc.
2153	V	135	3.0 cc.
2116	V	62	5.1 cc.
2118	V	25	3.5 cc.
2199	V	100	3.0 cc.
2206	V	71.5	4.9 cc.
1874	III	100	5.5 cc.
2290	B1	36.8	1.5 cc.
2133	B1	45.0	1.0 cc.
			Blood Volume[1]
2191	V	50	21.7%
2204	V	17.5	8.2%
2190	V	54	28.4%
2201	V	23.5	18.1%
2207	V	27.5	18.5%
2187	B1	75	23.0%
2135	B1	27.5	17.1%
2188	B1	18.5	15.6%
2132	B1	40.5	18.3%
2291	B1	25.0	18.8%
10 mice	Normal	17.5–30	

[1] Per cent body weight. All but last two values (2132, 2291) were obtained by the dye technique; the last two mice were exsanguinated and perfused.

with prothrombin value of 124.5% had a slight liver congestion. In 6 of the 10 mice with granulosa cell tumor tested, the prothrombin values were above the highest normal value of this series (106.5%). Thus hypervolemia itself seems to be associated with an elevated prothrombin level until secondary changes damage the liver and the prothrombin levels drop below normal.

(2) Water tolerance tests were normal in 5 of 6 mice with hypervolemia. The excretion ratios were as follows: 1.15, 0.86, 0.92, 1.30, 0.86 and 0.38.

(3) The blood glucose levels after 6 to 7 hours of starvation of 6 of 12 mice bearing these granulosa tumors but with unknown blood volumes were below 85 mg.%, the lowest value observed in normal

mice during this period. Eight of nine normal mice had blood glucose levels ranging from 105–130 mg.% after 6 to 7 hours starvation. If 105 mg.% is taken as the lower limit of normal then 9 to 12 mice bearing the granulosa cell tumor had low values while 5 to 12 mice bearing other tumors had low values.

(4) Sodium and potassium determinations on the plasma of hypervolemic mice yielded variable results and require further observations.

DISCUSSION

The marked hypervolemia observed occurs only in mice bearing transplanted granulosa cell tumors. This is a unique phenomenon thus far not noted in women with granulosa cell tumors or in mice with spontaneous or induced (non-transplanted) granulosa cell tumors of the ovary. However, in mice at least, the spontaneous or induced ovarian tumor does not attain the bulk of the transplanted granulosa cell tumor. Furthermore no blood volume determinations are available on women or mice with large spontaneous growths of this type.

The phenomenon is dormant for a long time in mice with progressively growing granulosa tumor and then it appears rapidly. This is a puzzling feature the understanding of which may furnish the key to this phenomenon. The ability of the animal to inactivate a hypothetical substance secreted by the tumor or to compensate for it may be overcome by the increasing amounts of this substance.

Relation to estrogens. The neoplastic granulosa cells here described produce estrogen although there does not appear to be a direct association between this property and the production of hypervolemia (Furth and Sobel, 1946). Preliminary experiments on sustained administration of large doses of stilbesterol and natural estrogens have failed to elicit hypervolemia. The granulosa cell tumor may well be capable of secreting other substances as precursors of the estrogenic material or perhaps an independent substance or a metabolite of substances secreted is responsible for the hypervolemia.

Hormonal regulations of blood volume. It is known that several steroids can cause retention of salt and water (Harrop, 1937; Thorn, Nelson and Thorn, 1938) and that desoxycorticosterone acetate causes an increase in blood volume (Clinton and Thoon, 1943). Various endocrine imbalances have been known to alter somewhat the blood volume. The plasma volume is decreased in myxedema (Gibson and Harris, 1939) and in adrenal insufficiency (Rowntree and Brown, 1929). It is increased in hyperthyroidism (Gibson and Harris, 1939). The increase in blood volume and the retention of water seen in liver disfunction may also be due to an endocrine imbalance (Labby and Hoagland, 1947).

The increase in blood volume found in these conditions is less severe than the hypervolemia of mice bearing granulosa cell tumors and differs quantitatively from it. An increase of the extracellular-

extravascular space is not part of hypervolemic process secondary to granulosa tumors. It is thus probable that the mechanism of the production of hypervolemia in mice bearing the granulosa cell tumor differs from the known physiological changes in body water and blood volume and is dependant on a hitherto unidentified principle.

The relation of hypervolemia to increased circulating albumin. In nearly all instances the plasma albumin concentration in hypervolemic mice is normal and therefore the amount of total circulating albumin is increased. This raises the question as to whether the hypervolemia caused an increase in circulating albumin or whether it is secondary to heightened plasma albumin synthesis. It is interesting to note in this connection that estrogens have been observed to increase plasma albumin (Levin and Leathem, 1942).

Starling's theory requires that a decreased or increased concentration of serum albumin be followed by a decreased or increased blood volume respectively so as to maintain the capillary blood pressure unchanged. A dynamic equilibrium exists between plasma protein and tissue proteins (Holman, Mahoney and Whipple, 1934); and an ever increasing rise in the amount of albumin in the blood might be expected to increase the blood volume.

There appears to be no information on the influence of repeated injections of excessive amounts of albumin on the plasma volume. Heyl, Gibson and Janeway (1943) have shown that a blood volume rise followed the single administration of a concentrated solution of human and bovine plasma albumin to men who were bled 10–20% of their total blood volume. The increase was in agreement with the values expected from in vitro studies of the osmotic pressure of these substances.

Elman and Davey (1943) obtained an increase in plasma protein after a week of transfusion of large quantities of plasma into dogs with dietary hypoalbuminemia. Both plasma albumin and globulin were increased, mostly the former. There was also a striking increase in plasma volume, so that the total circulating albumin doubled in all experiments. Holman (1942) also obtained increased plasma volume after repeated injections of homologous plasma into dogs.

No clinical condition is known to be associated with hyperalbuminemia. This would, however, be obscured by an increase in plasma volume and since blood volume determinations are not made systematically the hyperalbuminemia would be overlooked.

It needs emphasis that the material in the albumin fraction of the hypervolemic mice has not yet been shown to be albumin only and an electrophoretic study of the plasma is desirable.

Possible relationship to V.D.M. A puzzling feature of this hypervolemia is the selected and variable involvement of adrenals, liver and spleen. While the relative distribution of erythrocytes and plasma in various organs of the hypervolemic animal has not yet been deter-

mined, it is obvious from morphological data that by far the greater portion of the increased blood volume is in the liver. According to Maximow and Bloom (1944), the sinusoids of the adrenal cortex resemble those of the liver and it is possible that both respond to the same specific substance.

Grab, Janssen and Rein (1929) have shown that the injection of 1/100–1/20 mg. of adrenaline into the dog produced an immediate expulsion of blood from the liver amounting to 26–59% of the weight of that organ. The analogous behavior of the adrenal does not appear to have been reported. A vasodilator substance or one which inhibits the above effect of adrenaline upon the liver could be responsible for the congestive changes. This suggested a search for a substance similar to the *V.D.M.* of Schorr, Zweifach, and Furchgott (1945). These workers have assayed the tumors, blood and livers of the hypervolemic animals and their findings will be presented elsewhere. It appears from their studies that an excess of *V.D.M.* may come from the liver of the hypervolemic mouse but not from the tumor.

If a vasodilator substance is slowly elaborated by the tumor-bearing animal a secondary increase of blood volume can be anticipated (Bard, 1941). In the light of this hypothesis this hypervolemia is in essence a mild protracted shock.

The recent studies of Clark and associates (1947) have shown that malignant tumors in man are associated with a reduced blood volume. They did not test granulosa tumors. The concept presented above would contradict current ideas assuming that chronic shock is associated with a reduced blood volume.

Possible relation to the function of the hepatic sphincter. Moutner and Pick (1929) have demonstrated the presence of a sphincter in the hepatic vein which is constricted by histamine and released by adrenaline in the dog but not in the goat or cat.[8]

If the congestive changes were caused by sphincteral constriction of the hepatic vein it would be expected that congestion in the liver be of the central type and that there would be a marked passive congestion of the spleen and intestine. Although splenic congestion does occur with hypervolemia, it can be slight and mesenteric congestion is rare. Furthermore, the gonads and bone marrow, which are not in the portal system, are also slightly or moderately congested. The adrenals can be extremely congested in the presence of slight liver congestion.

SUMMARY AND CONCLUSIONS

Repeated blood volume determinations were made on mice bearing transplanted granulosa cell tumors. The blood volume begins to rise after the tumors have reached a moderate size. Once this process is set in motion the blood volume increases rapidly and consistently.

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[8] Liver congestion was not observed in 2 mice bearing subcutaneous pellets of 50 mg. of histamine dihydrochloride in 100 mg. of beeswax.

This hypervolemia is not accompanied by an increase in the extra-cellular-extravascular space.

The blood urea levels may be high or normal in the presence of advanced hypervolemia. The prothrombin values are frequently above normal, both liver and kidney seem to be functioning normally at onset of hypervolemia.

The plasma protein concentration may be normal and when it is reduced this is due to a decrease of plasma globulin concentration. The absolute amount of plasma albumin is increased and this increase roughly parallels the degree of hypervolemia. Further studies are needed to determine if this rise in albumins is the cause or consequence of hypervolemia and to establish the site of this albumin production.

ACKNOWLEDGMENTS

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EFFECT OF ULTRAVIOLET IRRADIATION ON BIOLOGICAL ACTIVITY OF α-ESTRADIOL BENZOATE[1]

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SMITH AND SMITH (1944, 1945, 1946, 1947) have advanced the theory that *in vivo* estrogen degradation products which are themselves relatively inactive on the vagina, may have certain other properties which have been attributed to estrogen *per se*, notably the effects upon the secretory activity of the anterior pituitary.

In this connection they investigated the pituitary stimulating ability of Westerfeld's lactone, an *in vitro* oxidative inactivation product of estrone. Although the lactone was only one-fifteenth as active as estrone with respect to vaginal estrus, it was reported to be more active than estrone in causing the release of luteinizing hormone from the pituitaries of immature female rats. It was also found to be quite active in reducing the transplantable pituitary gonadotrophic activity and in increasing the adrenal and pituitary weight of male rats. This activity was reported to be much greater than could be accounted for on the basis of the slight estrogenic activity of the lactone. Moreover, this activity was exhibited in either intact or castrate male rats, whereas estrone exhibited similar activity only in castrate male rats.

The Smiths interpret this difference as indicating that the lactone exerts its effect by directly stimulating the pituitary, whereas estrone acts indirectly through the formation of the lactone or some similar compound whose production is inhibited by testicular secretion. They point out that testosterone, progesterone and adrenal cortical extract all reduce the rate of formation of a non-estrogenic urinary estrogen degradation product which they find in amounts accounting for 10 to 60 per cent of the estrone administered to women. They found that this urinary component, although not made estrogenically active by simple acid hydrolysis, is, like Westerfeld's lactone, reactivated by zinc-hydrochloric acid hydrolysis.

Simpson and Williams (1946) and Bradbury (1947), on the other hand, have published results which do not appear to be in complete agreement with the work of Smith and Smith.

Figge (1943, 1945) irradiated an alkaline aqueous solution of estrone with unfiltered light from a quartz mercury arc. When assayed on ovariectomized rats, the irradiated estrone gave only occasional positive vaginal smears at doses up to 6.4 micrograms whereas the non-irradiated control estrone solution produced estrus in 100 per cent of the animals at levels of 1.6 micrograms and above. However, when 50 micrograms of the irradiated estrone was injected into intact female mice, estrus of 10 to 14 days duration ensued, or usually 2 to 3 days longer than in the controls which received a corresponding dose of non-irradiated estrone. When the solutions were again tested, this time on ovariectomized mice, the irradiated estrone was found to be "relatively inactive" as compared to non-irradiated estrone, although dosages were not stated. The apparent failure of the irradiated product to produce estrus in ovariectomized females while producing estrus in intact female mice was interpreted on the basis of the theory of Smith and Smith. That is, irradiation of estrone was assumed to have destroyed its direct vaginal stimulating activity while enhancing its ability to stimulate the pituitary to increased gonadotrophic activity.

The present work represents an attempt to investigate the possibility that ultraviolet irradiation of the estrogens, although decreasing their vaginal stimulating activity in ovariectomized animals, might enhance their effectiveness in the intact animal, by increasing their effectiveness as stimulants of pituitary gonadotrophic activity. In addition, mammary stimulating activity was also observed before and after irradiation.

Allen and Ellis in 1925 observed that ultraviolet irradiation of corn oil solutions of hog follicular extract destroyed their vaginal stimulating activity, but no mention was made of pituitary stimulating activity.

Butenandt et al., (1941, 1942, 1943) have extensively studied the chemistry of the ultraviolet irradiation of several of the steroid sex hormones. In the case of estrone they have identified in the irradiation mixture the 13-epi-isomer of estrone, to which they gave the name lumiestrone. They found this to be inactive in 10 microgram doses in the Allen-Doisy test, but gave no information regarding pituitary stimulating activity. The yield of this isomer was relatively small, and appreciable amounts of unchanged estrone were also recovered from the irradiation mixture.

METHODS

Two ml. of a sesame oil solution of α-estradiol benzoate[2] were placed in the inverted lid of a 10 cm. petri dish. The solution contained 0.33 mg. of the estrogen per ml. This was placed 12 inches from the mercury arc of an ultraviolet lamp (Hanovia, 110 volts, 60 cycles, 5 amps.) and irradiated for a period of 24 hours. During this process the temperature at the petri dish rose to 50°C. and the oil became very viscous but remained clear. Next,

[2] Courtesy of Schering Corporation, Bloomfield, N. J.

1.65 ml. of the oil was recovered and diluted with olive oil to give a concentration of 200 micrograms per ml. (figured on the basis of 330 micrograms per ml. of the irradiated oil recovered). This solution was still clear and somewhat viscous. From it weaker dilutions were made using olive oil. At 20 micrograms per ml. a fairly heavy flocculent precipitate resulted, which was very slow in settling out. At 10 micrograms per ml. the precipitate was lighter, at 2 micrograms per ml., barely detectable, and at 0.2 micrograms per ml., not visible. Such precipitate formation has not been observed to occur upon dilution of non-irradiated sesame oil solutions of this estrogen with olive oil.

The ovariectomized mice used for vaginal assay, with the exception of those indicated by an asterisk in Table 1, were primed with 0.1 micrograms of α-estradiol benzoate. Any animals that did not respond to this level of

TABLE 1. VAGINAL RESPONSE OF OVARIECTOMIZED FEMALE MICE TO α-ESTRADIOL BENZOATE BEFORE AND AFTER ULTRAVIOLET IRRADIATION

Total dose, micrograms	Estradiol benzoate		Irradiated estradiol benzoate			
	No. mice	% positive	No. mice	% positive	No. mice	% positive
10.	19	100*	14	100		
1.	16	100*	14	86	14	64*
0.5	17	94*	14	43	14	43*
0.1	178	81*	24	0		
0.08	16	81				
0.06	16	69				
0.05	16	56				
0.04	15	33				
0.02	14	0				
0.01	15	0				

* These figures obtained on unselected, unprimed animals. All other data obtained on primed animals that had responded with a positive smear to 0.1 microgram of α-estradiol benzoate.

estrogen were withdrawn from the assay groups. Ten to 14 days after priming the mice were used for assay. On day No. 1 a single injection in 0.05 ml. of oil was given. Smears were taken morning and evening on days 4 and 5.

To test the irradiated and non-irradiated estrogen solutions in intact female mice, groups of normal female mice were smeared regularly. After observing the vaginal smears for a number of days, the animals were injected. The incidence of positive vaginal smears per day before and after injection was tabulated.

For mammary growth assay intact male albino mice were used. In order to make the mammary assay as comparable as possible with the vaginal assay, the mice were given a single injection in 0.05 ml. of oil on day No. 1 and killed on days 5 or 7. Two groups were injected on days 1 and 5 and killed on day 9. The formation of growing end-buds on the mammary rudiments was taken as criterion for positive response. In the strain of mice used the males show a low incidence of spontaneous end-bud occurrence (3 out of 18 mice run as controls with these experiments).

RESULTS

As seen in Table 1, ultraviolet irradiation resulted in a loss of all but 10 per cent of the original vaginal stimulating activity present in

TABLE 2. VAGINAL RESPONSE OF INTACT FEMALE MICE TO α-ESTRADIOL BENZOATE BEFORE AND AFTER ULTRAVIOLET IRRADIATION

Day No.	Group I (21 animals)		Group II (17 animals)		Group III (24 animals)	
	Treatment	% of positive vaginal smears	Treatment	% of positive vaginal smears	Treatment	% of positive vaginal smears
1		43		12		12
2						25
3				18		0
4		14		12		42
5		14		24		42
6				18		33
7		7		29		33
8		19		6		4
9		24		12		12
10				24		33
11		24		29		37
12		29	0.1 µg. non-irradiated estrogen	12		37
13		10		18	0.1 µg. non-irradiated estrogen	17
14		14		29		12
15		14		65		50
16				24		71
17			0.1 µg. non-irradiated estrogen	12		62
18		19		12		26
19	0.1 µg. irradiated estrogen	29		35		13
20		19		76	0.1 µg. irradiated estrogen	13
21		10		59		17
22		24		24		9
23		24		12		9
24		14	0.1 µg. irradiated estrogen	12		26
25		5		35		22
26		14		18		
27		19		6		17
28		5		18		13
29		10		6		33
30		14				
31		10		12		
32				29		
33				25		

the oil solution, as judged by the amounts required before and after irradiation to give a positive vaginal smear in approximately 50 per cent of the ovariectomized mice.

To study the possibility of enhanced gonadotrophic stimulating activity, in intact female mice, the 0.1 microgram level was chosen. As seen from Table 1 this was the highest level of the irradiated product that could safely be given without causing direct vaginal stimulation as a result of residual estrogenic activity. If the 90 per

cent loss of direct vaginal stimulating activity was accompanied by conversion of the estrogen to a pituitary gonadotrophic stimulant, this level of irradiated estrogen should cause an increased incidence of positive vaginal smears in intact female mice. However, as seen in Table 2, 0.1 microgram of the irradiated estrogen caused no increase in the daily incidence of positive vaginal smears, whereas the same level of the non-irradiated estrogen caused a pronounced increase within 72 to 96 hours. Irradiation therefore decreased the vaginal stimulating activity of the estrogen in both intact and ovariectomized mice.

Comparison of the mammary stimulating activity of the irradiated and non-irradiated estrogen is presented in Table 3. The unevenly

TABLE 3. MAMMARY RESPONSE OF INTACT MALE MICE TO ULTRAVIOLET-IRRADIATED AND NON-IRRADIATED α-ESTRADIOL BENZOATE*

Total dose, micrograms	4 days				6 days				8 days			
	Estradiol benzoate		Irradiated estradiol benzoate		Estradiol benzoate		Irradiated estradiol benzoate		Estradiol benzoate		Irradiated estradiol benzoate	
	No. mice	% positive	No. mice	% positive	No. mice	% positive	No. mice	% positive	No. mice	% positive	No. mice	% positive
1.	10	90	10	50	10	80	10	60				
0.1	10	80	13	85	9	77	8	25				
0.05	11	36	13	54								
0.02									12	33	13	46
0.01	11	9	11	36	11	36	12	33				

* The formation of growing end-buds was considered a positive response. Male mice of the strain used show a low incidence of spontaneous end-bud occurrence. Two groups of 9 untreated controls showed 22 and 11 per cent positive respectively.

graded nature of the response to the irradiated estrogen makes evaluation of the results difficult. In general, at the higher dosage levels the irradiated estrogen appears less active than the non-irradiated estrogen. At the lower dosage levels the response to the irradiated estrogen appears to be as good as, or better than the response to non-irradiated estrogen. The spontaneous occurrence of a small percentage of "positive" mammary glands among the untreated control animals, however, detracts somewhat from the significance of the results on the lower dosage levels.

DISCUSSION

The present work confirms the reduced vaginal stimulating activity resulting from ultraviolet irradiation of estrogens. However, within the limits of the differences in techniques and materiel, it fails to support Figge's interpretation of enhanced pituitary stimulating activity as indicated by vaginal cornification in the intact animal. Figge's results may perhaps be explained on a dosage basis. The

dosage of 50 micrograms which he observed to cause vaginal cornification in intact mice was considerably greater than the dosages which were observed to be inactive in ovariectomized rats. In view of the work of Butenandt who always found unchanged estrone in his irradiation mixtures, it would appear that Figge's results can readily be explained on the basis of unchanged estrone.

Since the present work employed α-estradiol benzoate instead of estrone, it cannot be compared strictly to the work of either Figge or Butenandt. It is apparent, however, that any work ascribing pituitary stimulating activity to an estrogenically inactive irradiation product of an estrogen must either be done on a purified irradiation product (such as Butenandt's lumiestrone), or else must demonstrate quantitatively that the irradiation mixture possesses a pituitary stimulating capacity greater than can be accounted for on the basis of residual estrogenic activity.

SUMMARY

Ultraviolet irradiation of an oil solution of α-estradiol benzoate resulted in a loss of 90 per cent of its vaginal stimulating activity. It was not possible to demonstrate in the irradiated product an enhanced ability to stimulate pituitary gonadotrophic secretion as measured by vaginal cornification in the intact female mouse.

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HISTOLOGICAL OBSERVATIONS ON THE RELATION OF INSULIN TO THE DEPOSITION OF GLYCOGEN IN ADIPOSE TISSUE[1]

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THE ADIPOSE tissue of animals in normal nutritional balance contains no histologically demonstrable glycogen. However, Gierke 1906 found that glycogen is deposited in the fat cells of guinea-pigs refed after a period of fasting. The occurrence of glycogen in the fat of other animal species under similar experimental conditions has been confirmed by several European investigators (Arndt, 1926, Wertheimer 1928, Richter 1931, Eger 1938, 1942). Among these Arndt, using dogs, also reported a transient accumulation of glycogen in the fat cells after insulin administration. These old observations take on new interest in the light of recent advances in our knowledge of the biochemistry of adipose tissue (Hook and Barron 1941, Mirski 1942), and the relation of insulin to lipogenesis (Drury 1940, Stetten and Klein 1946). We have therefore undertaken to investigate, with an improved staining method, the deposition of glycogen in the brown and white adipose tissues of normal and diabetic rats, rats given insulin, and rats refed after a period of fasting.

MATERIAL AND METHODS

Forty-five male and female rats of the Long-Evans strain weighing 200–250 gms. were used. The deposition of glycogen in adipose tissue was studied in rats given food freely for 48 hours after a five-day period during which food was withheld. To determine the influence of a high intake of carbo‧hydrate on this process, some of the animals were given pure dextrose during the feeding period while others fed on their usual diet of Purina dog-chow which contains approximately 70 per cent carbohydrate absorbable as dextrose. The effect of insulin deficit on the deposition of glycogen was studied on rats made diabetic by intraperitoneal injection of 200 mg. per kilogram of alloxan monohydrate (Eastman) into animals fasted 48 hours. The effect of insulin excess was investigated on animals sacrificed 24 hours after receiving 5 units protamine-zinc-insulin (Lilly) subcutaneously. Interscapular brown-fat and the adjacent subcutaneous white-fat were removed together and fixed in a mixture of absolute alcohol, formalin and picric acid (Ross-

man's fluid). Sections were stained for glycogen by McManus' periodic acid technique. This method was originally devised as a stain for mucin (McManus 1946), but in applying it to a variety of tissues in this laboratory it was found that glycogen also stains very intensely. Deparaffinized sections are immersed in a 1% solution of periodic acid for five minutes, then transferred to Schiff's reagent for fifteen minutes. After treating for ten minutes in a solution of sulphurous acid, the sections are washed, then cleared and mounted in the usual sequence of alcohols, xylol, and clarite. Glycogen is stained an intense purple, mucin is somewhat less deeply stained, while collagen, elastic tissue, and a variety of other tissue components are stained pink. Either orange G or light-green are suitable counterstains, although ordinarily none is needed, owing to the pink background of non-specific staining produced by the Schiff reagent after periodic acid oxidation. Glycogen is easily distinguished from mucin by digestion of control sections with saliva. After periodic acid treatment, glycogen was often easily discernible in tissues which were negative or only faintly positive by other methods. The Bauer-Feulgen and Best's carmine stains were employed to some extent in the early part of this investigation, but they were soon abandoned in favor of the McManus periodic acid method because of its more uniform results, greater sensitivity, and more intense color. In a recently published comparative study of several methods for the demonstration of glycogen, Lillie (1947) also found that the use of periodic acid followed by the Schiff reagent was the method of choice. A comparison of the Bauer-Feulgen, Best's carmine, and McManus methods on sections from the same block is presented in figures 1, 2, and 3.

OBSERVATIONS ON GLYCOGEN IN ADIPOSE TISSUE

In addition to white or yellow adipose tissue which occurs in all well-nourished mammals, many species possess gland-like masses of so-called brown-fat located most commonly in the perirenal, interscapular, and axillary regions. In the rat, this tissue is readily distinguishable grossly from ordinary fat by its lobular arrangement and its color, which ranges from light tan to brown. There are also marked differences in microscopic appearance. In ordinary fat cells the cytoplasm is reduced to a thin layer enveloping a single large globule of neutral fat, while the nucleus is displaced to the periphery and flattened. The cells of brown fat contain multiple droplets of neutral fat in a rather abundant, coarsely granular cytoplasm. The nucleus is round and centrally situated.

Rats in normal nutritional state: Traces of glycogen were found occasionally in a few scattered cells of the brown fat of normal animals fed on dog-chow but ordinarily both types of adipose tissue were entirely devoid of stainable glycogen. In rats allowed to feed *ad libitum* on pure dextrose the fat was also negative (RC-7, RC-8).

Fasting and refeeding. When rats are permitted to feed after several days of fasting, the amount of glycogen in the fat has been found by chemical analysis to be greatly increased, reaching its maximum about the second day after the onset of feeding. Thereafter the glycogen content gradually falls off, nearing zero by the 4th day

(Tuerkischer and Wertheimer, 1941). These findings were borne out by preliminary histological studies on the fat of animals sacrificed after various periods of refeeding.'The majority of the observations reported under this heading was made therefore on rats fasted for five days, then given access to food for forty-eight hours. Under these experimental conditions glycogen was regularly found in conspicuous amounts in both types of fat.

In the white fat, glycogen occurred in the form of coarse, irregular granules or aggregates around the entire circumference of the thin layer of cytoplasm between the cell membrane and the contained fat vacuole (fig. 4).

In white fat the film of protoplasm around the periphery of the cell is so thin that it is not discernible in ordinary histological sections and histologists have been obliged to devise special methods to demonstrate its existence. These methods either involve the use of hypotonic solutions to cause swelling of the layer of protoplasm by inbibition of water (Bremer, 1938), or take advantage of the fact that some fat cells will store trypan-blue in their rim of cytoplasm after repeated injections of the dye over a long period (Dogliotti, 1928). These relatively elaborate and unphysiologic methods are no longer necessary, for when fat from a rat under the stated conditions is stained by the periodic acid technique, the presence of a thin film of cytoplasm around the periphery of the fat droplet is clearly revealed by the presence of glycogen. This observation is of further interest because it serves to reemphasize the fact that a layer of cytoplasm may be thinned out to the point of invisibility with the optical microscope and nevertheless retain the functional capacity for carrying out complex biochemical processes such as the synthesis of glycogen.

Brown fat is known to have a higher rate of oxygen consumption than white fat (Fleischmann 1929, Hook and Barron 1941) and recent studies using radioactive phosphorus have demonstrated that it also has a much more rapid phosphorus turnover (Littrell, Martin and Hartman 1944). A difference in the physiological activity of the two adipose tissues is also apparent in their response to fasting and refeeding. Although qualitatively the responses of the two kinds of fat were usually the same, glycogen was found to appear in distinctly larger amounts in the brown fat where it occurred as an abundant granular deposit in the cytoplasm between fat droplets. The cells of brown fat were not all equally active, for cells heavily laden with glycogen were often found adjacent to cells which contained none or only very small amounts (Fig. 5 and 6). Differences in amount of glycogen from one animal to the next were easier to evaluate in the brown fat because of its more abundant cytoplasm and greater content of glycogen. Animals are compared in table 1, therefore, on the basis of the amount of saliva-digestible, Schiff-positive material found in their interscapular brown fat. Occasional animals (viz. R-5

TABLE 1. THE GLYCOGEN CONTENT OF BROWN ADIPOSE TISSUE

	Animal number	Rat Chow ad libitum	Dextrose ad libitum
A. Control rats	RC-1	0	
	RC-2	+	
	RC-3	0	
	RC-4	0	
	RC-5	+	
	RC-6	0	
	RC-7		0
	RC-8		0
B. Rats fasted 5 days, then fed 48 hours	R-1	+++	
	R-2	+++	
	R-3	+++	
	R-4	++++	
	R-5	++	
	R-6	+++	
	R-7	++++	
	RS-1		++++
	RS-2		+++++
	RS-3		++
	RS-4		+++++
C. Rats on forced feeding by stomach tube.	RH-1		++ (300)*
	RH-2		+++ (368)
	RH-3		++++ (466)
	RH-4		++++ (310)
D. Rats killed 24 hrs. after receiving protamine-insulin:	RI-1	++	
	RI-2	++	
	RI-3	+	
	RI-4	+++	
	RI-5	++	
5 units	RI-6	++	
	RI-7	+++	
	RI-8	++	
	RIS-1		++++
	RIS-2		+
	RIS-2		++++
	RIS-4		++++
	RI-9	+++	
10 units	RI-10	+++++	
	RI-11	++++	
	RI-12	+++++	
E. Rats with alloxan diabetes	RD-1	0 (562)*	
	RD-2	0 (390)	
	RD-3	0 (284)	
	RD-4	0 (262)	
	RD-5	0 (176)	

* Blood-sugar level in milligrams per 100 cc.

and RS-3) showed more glycogen in the white adipose tissu̶e̶ t̶h̶a̶n̶ i̶n̶
the brown but these were quite exceptional.

When rats had access only to dextrose during the r̶e̶f̶e̶e̶d̶i̶n̶g̶
greater amounts of glycogen were found in their fat t̶h̶a̶n̶ t̶h̶o̶s̶e̶ t̶h̶a̶t̶
fed on dog-chow (figs. 8 and 9). Hence the quan̶t̶i̶t̶y̶ o̶f̶ g̶l̶y̶c̶o̶g̶e̶n̶
deposited appears to depend to some extent upon t̶h̶e̶ c̶a̶r̶b̶o̶h̶y̶d̶r̶a̶t̶e̶
content of the diet.

Since adipose tissue is specialized for the storage of nutrients, it might be supposed that its cells were able to store carbohydrate as well as fat during a period of alimentary hyperglycemia such as might be expected to result from voracious eating after a prolonged fast. The effect of hyperglycemia was therefore investigated in rats which were force-fed large amounts of pure dextrose and in rats with alloxan diabetes.

Alimentary Hyperglycemia: Hyperglycemia was produced in two normal rats by giving a 50% solution of dextrose in water by stomach-tube. Four feedings of 2 cc. were given in the first 24 hours and 5 feedings of 4 cc. in the second, a total of 14 gms. of dextrose in 2 days. Blood sugars on the second day were 300 and 368 mgs. percent. Sections of the brown fat of these animals contained considerable glycogen (RH-1 and RH-2). The adjacent white fat was entirely negative. Two other rats which received 20 gms. of dextrose by stomach-tube in 2 days (RH-3 and RH-4) had blood sugars of 310 and 466 mgs. per cent when sacrificed and showed large amounts of glycogen in the brown fat and also a moderate amount in the white fat.

Alloxan diabetes: In 2 rats diabetic for 4 to 6 days (RD-1 and RD-2) with blood sugar levels of 390 and 562 milligrams per 100 cc. of blood, the fat was negative for glycogen (fig. 10). Since diabetic rats do not tolerate prolonged fasting, 4 animals (RD-3–RD-6) were fasted 2 days and then injected with alloxan. Food was withheld for 2 more days. One animal succumbed. The three which survived the 4 day period of fasting were then allowed to feed ad libitum for 48 hours (RD-3, 4, 5). Their blood-sugar levels were somewhat lower than those of the other diabetic animals probably because appetite was impaired by the ketosis associated with fasting. The fat of these rats was also devoid of glycogen. Thus neither hyperglycemia nor fasting and refeeding causes glycogen to appear in the fat of diabetic rats. Hence the occurrence of glycogen in adipose tissue, like the deposition of glycogen in liver and muscle, appears to depend upon insulin.

Insulin administration to normal rats. Richter 1931 was unable to demonstrate glycogen by histological methods in the fat-cells of

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EXPLANATION OF PLATE 1

FIGS. 1, 2, 3. Parallel sections from the same block of adipose tissue stained by three commonly used methods for the demonstration of glycogen. Fig. 1.—Bauer-Feulgen method, counterstained with light green. Fig. 2.—Best's carmine stain, counterstained with hematoxylin. Fig. 3.—Periodic acid-Schiff reaction, no counterstain. All three photomicrographs were made with a Wratten B filter and given identical exposure. ×160.

FIG. 4. Ordinary white adipose tissue of a rat. The glycogen appears as coarse granules of varying size in the narrow rim of cytoplasm around the periphery of the fat vacuole. ×440.

FIG. 5. Interscapular brown fat of a rat. Coarse granular deposits of glycogen are found in the cytoplasm between fat vacuoles. ×440.

FIG. 6. Interscapular brown fat of a rat given insulin. Many cells are very heavily laden with glycogen while adjacent cells may contain little or none. ×440
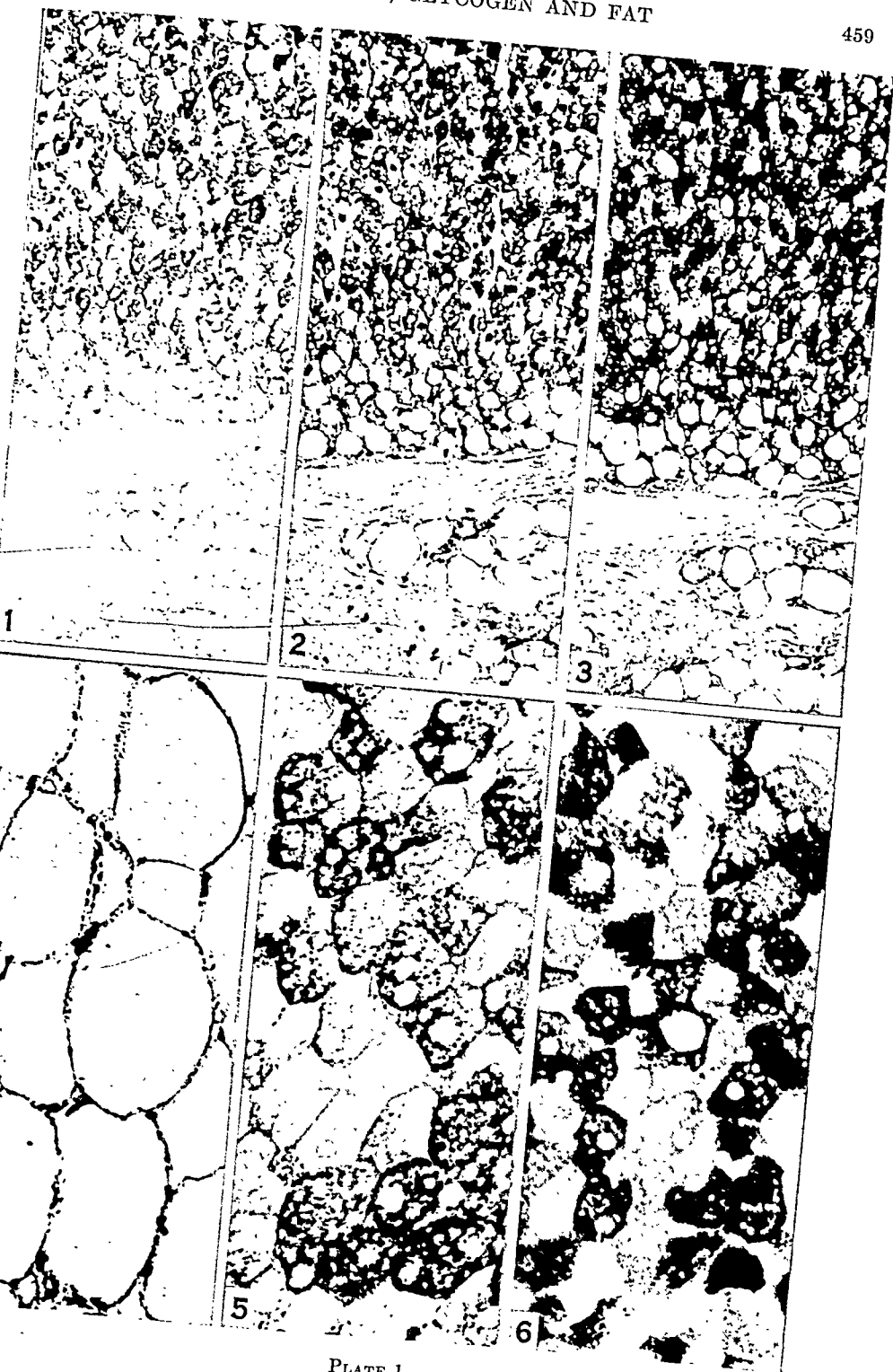
PLATE 1

10
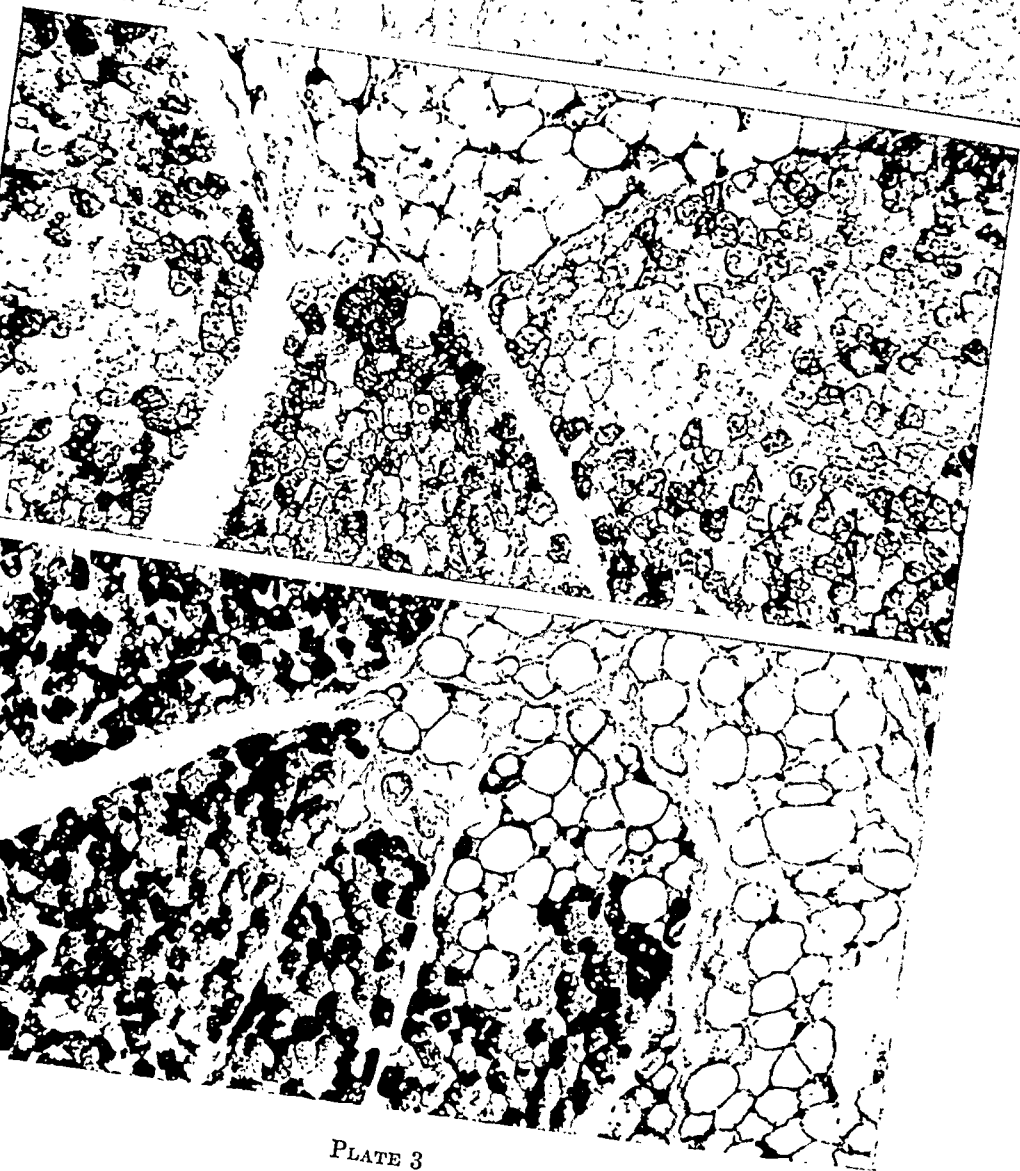
PLATE 3

injected at a distance. Ten rats were divided into pairs of the same weight. One of each pair received a subcutaneous injection of insulin directly over the interscapular fat, the other received the same dose under the skin of the abdomen. Three of the ten pairs of rats received 5 units of protamine-zinc-insulin. With this dose the differences between those injected near the interscapular fat and those injected on the abdomen were inconclusive. In one pair there was definitely more in the brown fat which was at the site of the injection. A second pair showed no significant difference. In a third pair the animal injected on the abdomen appeared to have more glycogen in its interscapular fat than the one injected interscapularly. The latter however showed a very irregular distribution of glycogen, some areas at the periphery of the fat-body (perhaps nearest the site of injection) contained a large amount of glycogen while the remainder showed relatively little. Two other pairs of rats were injected in the same manner with 10 units of protamine-zinc-insulin. With this larger dosage the 2 animals injected in the interscapular region had distinctly more glycogen in the interscapular fat than did the animals injected on the abdomen. The results of these few experiments were not sufficiently consistent to warrant a stronger statement than that they suggest that insulin does have a specific local effect upon fat cells.

The foregoing observation is of interest in view of the clinical observation that diabetic patients occasionally show extensive changes in the subcutaneous fat at the site of insulin injection—a condition commonly called insulin lipodystrophy. In the region involved, areas of fat atrophy and areas of hypertrophy may intermingle. In juvenile patients lipohypertrophy may predominate. Attempts to produce insulin lipodystrophy in normal experimental animals have failed and a specific local effect of insulin in the etiology of this condition has therefore been denied (Reed, Anderson & Mendell 1920, Goldner 1943). The dosage used for daily injection in those studies were based on human dosage per unit of body weight and may have been far too small (0.6 units in rats and 0.01 units for mice). Rats and mice with access to food will tolerate at least 100 times these doses. Our findings on rats indicate that large doses of insulin probably do have a specific local effect on fat cells which may have a bearing upon the problem of insulin lipohypertrophy.

EXPLANATION OF PLATE 3

FIG. 10. Interscapular brown fat (right) and adjacent subcutaneous white fat (left) of a diabetic rat (RD-1) with a blood sugar 562 mg/percent. Both types of adipose tissue are negative for glycogen. Stained for glycogen by the periodic acid-Schiff method. ×160.

FIG. 11. Interscapular brown fat and adjacent white fat of a rat (RI-2) fed dog chow ad libitum for twenty-four hours after receiving a single subcutaneous injection of 5 units protamine-zinc-insulin. Glycogen 2 plus. Periodic acid-Schiff method. ×160.

FIG. 12. Brown fat and white fat of a rat(RIS-1) fed dextrose ad libitum for twenty four hours after receiving a single dose of 5 units of protamine-zinc-insulin. Glycogen 4 plus. Periodic acid-Schiff method. ×160.

DISCUSSION

Numerous physiological investigations have established that in normal animals large doses of insulin tend to inhibit accumulation of glycogen in the liver but cause a decided increase in the glycogen of muscles (Barbour, Chaikoff, McLeod and Orr 1927, Bridge 1938, Swensson 1945). In experiments to determine the effects of exogenous insulin, it has been customary to analyze liver and muscle on the assumption that these were the only "active tissues" involved. It is now apparent from the present histochemical observations, that insulin causes an increase in the glycogen content of adipose tissue as well as muscle. The role of glycogen in muscle physiology is well known, but the significance of glycogen deposited in fat has yet to be elucidated.

Von Gierke 1906 interpreted the glycogen he found in adipose tissue as an intermediate product in the transformation of dietary carbohydrate to depot fat. He thought the occurrence of glycogen in fat-cells during refeeding constituted morphological evidence that this conversion could take place in the fat-cells themselves. These observations attracted little attention because they were contrary to accepted biochemical theory and they were reported at a time when the fat depots were believed to be metabolically inert. Some twenty years later, however, with the discovery that isolated adipose tissue consumes oxygen (Hoffman and Wertheimer 1927), investigations of adipose tissue for evidences of metabolic activity were undertaken with renewed interest. The occurrence of glycogen in the fat of animals fed a high carbohydrate diet after preliminary fasting was confirmed by histological observations (Arndt 1927, Richter 1931) and by chemical analysis (Wertheimer and Hoffman 1927, Scoz 1929, Hausberger and Neuenschwander-Lemmer 1939). In vitro measurements of the respiratory metabolism of fat from animals under these same conditions were found to yield values for respiratory quotient (RQ—Vol. CO_2 output/vol. O_2 intake) which often exceeded unity (Ruska and Quast 1935, Henle and Szpingier 1936, Felix and Eger 1938, Mirski 1942). Since the highest RQ found from combustion of any foodstuff is 1.0 (carbohydrate), higher values are thought to mean that oxygen is being derived from some source other than the respired air. The transformation of carbohydrate to fatty acid would liberate oxygen. It is therefore assumed that values for RQ greater than 1.0 signify the conversion of carbohydrate to fat. Based on this physiological evidence and the earlier morphological and chemical observations of glycogen in fat, the belief became rather widely held in Europe that adipose tissue is capable of synthesizing fat from carbohydrate and that the deposition of glycogen in fat-cells is in some way related to this process.

Investigators in this country, for the most part unfamiliar with the European literature, generally adhered to a more conservative view of the functional capacities of adipose tissue. The new insight

into the intermediary metabolism of dietary fat which has recently been gained through the use of isotopes, has nevertheless forced us to abandon the traditional concept of depot fats as static reserves. The brilliant work of Schoenheimer 1942 and his coworkers has revealed that stored fats are constantly involved in a variety of complex reactions of synthesis, degradation and interconversion. It is still the consensus of opinion, however, that these elaborate molecular reorganizations of depot fat as well as carbohydrate-fat synthesis, take place in the liver and that the products are continuously trans-ported between liver and fat depots by the blood stream. Although adipose tissues are not generally believed to participate in metabolic processes to any important extent, the possibility that they have some part to play in the synthesis and degradation of fatty-acids has not been excluded. The recent studies of Tepperman, Brobeck and Long (1943) indicate that under certain circumstances extrahepatic mechanisms for the conversion of carbohydrate to fat may assume considerable importance. Studying the respiratory exchange of rats with hypothalamic lesions, these investigators found that such ani-mals showed a much greater elevation of R. Q. during glucose absorp-tion than did controls. This suggested that the obesity developed by rats with experimental lesions in the hypothalamus may be the result of a biochemical adaptation by which these animals are enabled to store a greater-than-normal proportion of their food as depot-fat. This change in food utilization is probably brought about by the altered feeding habits of animals with hypothalamic lesions, for Tepperman and his co-workers found a similar elevation of R.Q. in intact animals trained to take their entire ration in only 3 hours of each day. Eviscerate preparations of rats trained in these feeding habits, also exhibited values for R.Q. which were significantly higher than those of the controls. Moreover, when these liverless animals were given added insulin the average R.Q. was greater than one. This was interpreted by Tepperman, Brobeck and Long as evidence for the synthesis of fat by an extrahepatic mechanism which is facilitated by insulin. The tissue in which the synthesis occurred was not identified.

In this connection it is interesting to note that the alteration of feeding habits which Tepperman and his coworkers induced in rats by training consisted essentially of alternating short periods of fasting and rapid feeding and is therefore similar in principle to one of the conditions we have employed to cause glycogen deposition in fat-cells. It has already been shown that isolated adipose tissue from animals fed after previous fasting has an R.Q. greater than one (Wertheimer, 1927; Mirski, 1942). It is suggested therefore that the extrahepatic synthesis of fats which was postulated by Tepperman *et al.*, may take place in the adipose tissue.

When insulin is administered to a normal rabbit, a large increase in hepatic lipogenesis is observed (Stetten and Klein, 1944). The work of Tepperman and others discussed above, suggests that extra-

hepatic lipogenesis may also be increased by insulin injection. In the present investigation insulin administration has been shown to cause glycogen to appear in the adipose tissues of normal rats, particularly in the so-called brown fat. In diabetic rats, on the other hand, where the rate of fatty acid synthesis is known to be only about 5% of normal (Stetten and Boxer), glycogen was never found in the fat cells. Hence both lipogenesis and the occurrence of glycogen in adipose tissue are promoted by insulin excess and prevented by insulin deficit. Inasmuch as the deposition of glycogen in adipose tissue only occurs under experimental conditions, which are known to favor the rapid conversion of dietary carbohydrate to depot fat, it is reasonable to suppose that the glycogen is in some way involved in lipogenesis. The role glycogen might play in the synthesis of fat is somewhat obscure, but it is possible that the local oxidative breakdown of glycogen may provide both a source of energy and structural units for the synthesis of fatty acids in the adipose tissue itself. The transformation of carbohydrate to fat in the depots may not take place to any great extent in normal animals in nutritional balance. It does seem likely, however, that this extrahepatic mechanism for the formation of new fat may assume considerable importance during the period of recovery from a prolonged fast, after ingestion of excessive amounts of carbohydrate, and also in animals receiving a large dose of insulin.

If we are correct in assuming that the glycogen in adipose tissue is related to the conversion of carbohydrate to fat, then it would appear that more fat is synthesized from carbohydrate in brown adipose tissue than in the white. This might be expected to result in a different chemical composition of the fats in the two tissues inasmuch as fats synthesized from carbohydrate are more highly saturated than those derived from other sources (Anderson and Mendel 1928). No values are available for the iodine number of the fats in the brown adipose tissue of the rat but there is some indirect evidence suggesting that brown fat is more highly saturated than ordinary adipose tissue. Rats raised on a vitamin E deficient diet containing 20% cod-liver oil show a progressive accumulation of acid-fast pigment in their white fat depots but the brown fat shows no significant alteration (Mason, Dam, and Granados, 1946). This pigment is thought to consist of polymerized peroxides of long-chain unsaturated fatty acids. The fact that the brown adipose tissue is spared in this process is consistent with the thought that its fats are made chiefly from carbohydrate and are therefore relatively highly saturated. Application of osmic acid staining to both types of adipose tissue provides additional evidence of such a difference in chemical composition. Oleic acid and its compounds are blackened by the reduction of osmium tetroxide to osmium dioxide. Compounds of palmitic and stearic acids, being saturated, are stained only when exposure to osmic acid is followed by treatment with 70% alcohol which changes it to osmium hydroxide—a process called secondary staining. Thus when the secondary staining is omitted the amount of blackening of a fat with osmic acid is a

rough indication of its degree of unsaturation. Brown fat is not blackened by this treatment while the adjacent white fat is. It is suggested, therefore, that the neutral fats in brown adipose tissue are more highly saturated.

SUMMARY

The deposition of glycogen in adipose tissue has been investigated by histochemical methods in normal and diabetic rats, rats given insulin and rats refed after a period of fasting.

The fat cells of normal rats are ordinarily devoid of stainable glycogen. After the administration of a single large dose of protamine-zinc-insulin to normal rats and also during the refeeding of fasted animals, glycogen was regularly present in conspicuous amounts. Under both of these experimental conditions glycogen was much more abundant in the cells of the interscapular and perirenal brown fat that it was in the subcutaneous white fat. Glycogen was invariably absent from the adipose tissues of diabetic rats.

The significance of glycogen in adipose tissue has been discussed in relation to the extrahepatic synthesis of fats from carbohydrate.

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PLASMA INORGANIC IODIDE, A CHEMICAL REGULATOR OF NORMAL THYROID FUNCTION[1]

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IN AN EARLIER communication (Wolff and Chaikoff, 1948) it was shown that the administration of large amounts of iodine temporarily inhibits the capacity of the *normal* thyroid gland to bind iodine organically. This inhibition appeared to be related to the level of plasma iodine. So long as the concentration of plasma iodine exceeded 20–35γ per cent, no organic binding of iodine occurred in the gland, and only when the concentration fell below this critical range did the gland resume its function of depositing iodine in an organic form. On the basis of these findings it was postulated that the level of plasma iodine is part of a homeostatic mechanism governing hormone synthesis in the normal gland. Thus, when a large amount of iodine is ingested, the prevention of its deposition as organically bound compounds in the thyroid gland keeps the ingested iodine circulating as inorganic iodide, in which form it is readily excreted by the kidney. In this way, it was argued, the formation of toxic amounts of the thyroid hormone is prevented.

In the experiments described above, the inhibition of thyroid function was temporary, lasting some 8–17 hours, i.e. during the time when the concentration of plasma iodine remained well above 35γ per cent. The experiments described here provide additional proof that plasma iodine, probably by influencing the level of inorganic iodide in the gland, is an inhibitor of normal thyroid function. It is shown in the present study that the period of inhibition of thyroid function imposed by high iodine administration can be prolonged by preventing the escape of the administered iodine by excision of the kidneys.

EXPERIMENTAL

Long-Evans rats ranging from 171 to 240 gms, were used throughout. The animals were fed until the time of the operation but afterwards were permitted access to water only. The rats were first injected intraperitoneally with 6 mg. of sodium pentobarbital and were then completely anesthetized with ether. The kidneys were exposed by a lateral incision and the perirenal fat peeled off, care being taken

to avoid damage to the adrenals. The ureter and renal vessels were then tied close to the hilum and the kidneys removed. After the operation the animals were kept in a warm room (28–30°C.). Approximately 1–2 hours after they had recovered from the anesthesia, they were injected with I^{127} labeled with I^{131} and sacrificed at various intervals thereafter. Blood was removed by heart puncture, and total plasma iodine was determined for each rat on 2 cc. of plasma, as de-

TABLE 1. The Uptake of Iodine by Thyroids of Nephrectomized Rats Following Injections of 10 and 500γ of I^{127} as KI and Their Conversion to Diiodotyrosine and Thyroxine

Time after I^{127} injection	10γ I^{127} Injected				500γ I^{127} Injected			
	Total amount of injected I^{127} found in thyroid	Per cent of injected I^{127} found in thyroid as		Plasma total iodine*	Total amount of injected I^{127} found in thyroid	Per cent of injected I^{127} found in thyroid as		Plasma total iodine*
		In-organic	Or-ganic			In-organic	Or-ganic	
hours	γ			γ per cent	γ			γ per cent
4	0.87	11	89	12.3	4.6	99	1.0	178
4	1.5	5	95	13.2	7.5	99	0.39	200
4	1.4	7	93	12.8	5.5	99	0.93	215
10	2.4	3	97	8.9	12.5	99	0.36	225
10	0.91	16	84	8.9	7.0	99	0.84	230
10	0.70	11	89	9.5	11.5	99	0.64	150
18	2.9	4	96	10.5	6.3	99	1.3	161
18	2.0	5	95	12.4	8.9	99	0.79	313
18	1.9	6	94	15.0	2.6	96	3.8	195
26	1.4	3	97	8.8	7.9	99	1.4	185
26	1.9	6	94	9.2	7.6	99	0.92	306
26	2.4	3	97	9.2	5.8	98	2.1	146
32	5.5	2	98	8.9	9.2	99	0.67	105
32	4.1	2	98	9.5	6.8	97	2.9	173
32	4.3	2	98	8.9	8.0	95	4.9	225
40	4.7	2	98	8.5	1.8	74	26	218
40	3.8	2	98	8.0	9.9	96	3.7	180
40	4.4	2	98	8.8	3.9	81	14	200

* At time thyroids excised.

scribed by Taurog and Chaikoff (1946). The gland iodine was separated into organic and inorganic fractions as previously described (Wolff and Chaikoff, 1948).

RESULTS

The rats were injected with either 10 or 500γ of I^{127} as KI one to two hours after removal of both kidneys and groups of them sacrificed at intervals of 4, 10, 18, 26, 32, and 40 hours after the injection. Values for total plasma iodine and for the amounts of injected I^{127} recovered in the gland are recorded in table 1. The amounts of injected I^{127} present in the inorganic (trichloroacetic-acid soluble) or the organic (trichloroacetic-acid insoluble) fractions of the gland were obtained by multiplying the numerical proportion of the injected I^{131} recovered in the respective fraction by the gamma of I^{127} injected.

Rats Injected with 10γ of I^{127}—The highest concentration of I^{127} found in the plasma of rats that had been injected with 10γ of I^{127} was 15γ per cent. Since the kidney is the main pathway of excretion

for inorganic iodide (Keating, Power, Berkson, and Haines, 1947; Nelson, Palmer, Park, Weymouth, and Bean, 1947; Spector, Mitchell, and Hamilton, 1945; Perlman, Chaikoff, and Morton, 1941), the finding of plasma-iodine values of 8–9γ per cent in nephrectomized rats as late as 40 hours after the injection is not surprising.

In this experiment the amounts of the *injected* I¹²⁷ recovered in the thyroid gland increased with time. At the later intervals about 4γ or 40 per cent of the injected dose were found in the gland. *At all intervals studied, approximately 90 per cent of the newly accumulated iodine was organically bound.*

TABLE 2. RELATION OF THE LEVEL OF PLASMA IODIDE TO ORGANIC BINDING OF IODINE BY THE THYROID GLAND

(All values are the averages of the results recorded in table 1)

Interval after injection of I¹²⁷ as KI hours	Plasma iodine γ per cent	Injected I¹²⁷ recovered in gland		
		Total γ	Organically bound	
			Amount γ	Per cent of total
4	13	1.3	1.2	92
	198	5.8	0.04	0.69
10	9	1.3	1.2	92
	202	10.3	0.06	0.58
18	13	2.3	2.2	96
	223	5.9	0.08	1.4
26	9	1.9	1.8	95
	212	7.1	0.10	1.4
32	9	4.6	4.5	98
	168	8.0	0.22	2.7
40	8	4.3	4.2	98
	199	5.2	0.46	8.9

Rats Injected with 500γ of I¹²⁷—The levels of plasma iodine in these rats were always in excess of 100γ per cent and in some were over 300γ per cent. The concentration of plasma iodine remained fairly constant during the entire period of observation (average, 210γ per cent).

Larger amounts of I¹²⁷ were recovered from the glands of the rats that received 500γ than from those of rats injected with 10γ. But in this experiment practically none of the newly accumulated I¹²⁷ was organically bound. For example, in the rats that were sacrificed 26 hours after the injection of 500γ dose, from 5.6 to 7.9γ of the injected I¹²⁷ was found in the gland; yet only 0.07–0.12γ or at most 2 per cent of this iodine was organically bound.

DISCUSSION

In the present investigation 2 plasma levels of iodine, one in the neighborhood of 10γ per cent, the other of 200γ per cent, were estab-

lished and maintained for a period of 40 hours by a single injection of KI into nephrectomized rats. As shown in table 2, which summarizes the results of these experiments, so long as plasma iodine remained in the neighborhood of 200γ per cent, the gland failed to convert iodine to organic forms; even after 40 hours, only 9 per cent of the injected I^{127} found in the gland had been converted to organic forms. That the thyroid gland of the nephrectomized rat retained its capacity to bind iodine organically during the entire period of observation is brought out in the experiments in which the levels of plasma iodine were kept below 15γ per cent. More than 90 per cent of the iodine found in the thyroids of these rats was organically bound. The present investigation thus shows that the period of inhibition of organic binding by the thyroid gland can be extended at will·by maintenance of a high plasma-iodine level.

It was pointed out earlier that the inhibition of organic binding of iodine by the gland offers a rational explanation for the response of the thyrotoxic patient to iodine treatment (Wolff and Chaikoff, 1948). This view is fully supported by the results of the present investigation. Since the findings in the normal and nephrectomized rats show that the gland is inhibited only so long as the plasma concentration of iodine is kept high, it is suggested that the failure to maintain high enough levels of plasma iodine may account for the exacerbation of the hyperthyroid state sometimes noted during the treatment of patients suffering from hyperthyroidism.

SUMMARY

The relation of the level of plasma inorganic iodide to the organic binding of iodine in the thryoid gland was investigated. A single injection of 500γ of iodide into bilaterally nephrectomized rats made possible the maintenance of a plasma iodine level of approximately 200γ per cent for a period of 40 hours. Although the injected iodine readily entered the gland, practically none of the newly accumulated iodine was converted to organic forms even after 40 hours. On the other hand, when 10γ of iodine were injected into nephrectomized rats, the level of plasma iodine remained low enough (below 15γ per cent) to permit incorporation of nearly all the newly accumulated iodine into organic forms. The significance of these findings for iodine treatment of thyrotoxic patients is pointed out.

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THE EFFECT OF PARTIAL HEPATECTOMY ON THE *IN VIVO* ACTIVATION OF TRIPHENYL-CHLOROETHYLENE[1]

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In 1941 Emmens published the first of a series of extensive studies on the biologic activity of synthetic and natural estrogenic materials. In his early experiments he compared the ability of a wide series of compounds to produce vaginal cornification in spayed mice when given either by subcutaneous injection or by direct application to the vaginal mucosa. These studies yielded results of singular interest, in that the materials studied could rather easily be divided into two classifications: (1) those compounds which required only a small fraction of the subcutaneous dose when the material was given intravaginally; and (2) those compounds which required essentially the same amount of material by either route of administration. All the natural estrogens and their esters which were studied fell into the first group, which Emmens then termed the "estrogens." Diethylstilbestrol also fell into this first group. Triphenylethylene, on the other hand, fell into the second group, which Emmens termed the "proestrogens." The name of this second group was chosen because it appeared to Emmens that the organism had to convert these materials into estrogenically active forms before local activity could be achieved.

Further proof that the organism converted "proestrogens" to "estrogens" with local action was obtained (Emmens, 1942b) when the problem was studied in spayed mice with two separate vaginal sacs. These were prepared by the method of Robson and Adler (1940), who demonstrated that if natural estrogens are instilled locally into one of the vaginal sacs, it alone is cornified. In contrast to this, Emmens was able to show that if "proestrogens" are instilled locally in sufficient amount to cornify one sac, then the other is also cornified.

Stroud (1940) showed that if diphenyl-hexadiene (a "proestrogen") is given to rabbits, a small amount of a phenolic material, probably 4:4'-dihydroxy-ν:δ-diphenyl-β:δ-hexadiene (an "estro-

gen"), is excreted in the urine. Accordingly, Emmens (1943), studying the S/L (Systemic to Local) ratio of urinary extracts obtained from guinea pigs given various "estrogens" and "proestrogens," observed that if estrogens are administered, the urine has a high S/L ratio but not as high as the administered compound. If certain "proestrogens" are given, the urine possesses an S/L ratio which is much greater than that of the administered material. However, some "proestrogens" failed to increase urinary estrogen excretion. This seems to indicate that the urinary excretion products after "proestrogen" administration consist largely or wholly of "estrogens".

Applying our method of intrasplenic injections to the study of the problem (Segaloff, 1944), we observed that if triphenylethylene was injected intrasplenically with the spleen *in situ*, less material was required for vaginal estrus than if the material was injected subcutaneously or into a spleen divorced from the portal circulation. These observations led us to believe that the "proestrogens" are converted in the liver to estrogenically active materials. Emmens (1942a) noted that triphenylchloroethylene possesses estrogenic activity. However, since by our techniques the activation of triphenylchloroethylene was greater than triphenylethylene and its estrogenic activity was also so much greater, it was selected for further study.

All the "estrogens" except triphenylchloroethylene, which we had studied by our technique (Segaloff, 1943, 1944), showed pronounced inactivation in the liver. In addition to this, we (Segaloff, 1946) noted that partial hepatectomy greatly decreased the hepatic inactivation of α-estradiol. Accordingly, studies were undertaken to determine whether partial hepatectomy would reduce the degree of activation of triphenylcholoroethylene.

MATERIALS AND METHODS

Young adult female rats obtained from Maguran Farms were spayed 3 or 4 weeks prior to the study. Fourteen days after priming with 50 micrograms of estrone in 0.1 cc. of peanut oil injected subcutaneously partial hepatectomy was performed, under ether anesthesia, as in our previous studies. While the animals were still anesthetized, the test injection of triphenylchloroethylene in 0.05 cc. of 5% benzyl alcohol in sesame oil was made either intrasplenically or subcutaneously. Vaginal smears were taken with a moistened cotton-tipped toothpick, 48, 54, 60 and 72 hours after the injection. The smears were air dried and stained with hematoxylin and eosin. A smear which showed only cornified epithelial cells at one or more of these times was classified as representing a positive vaginal estrus response for that animal. Twenty or more animals were employed at each level of hormone administration.

RESULTS

The results are presented in Table 1. The results in the intact animals are in essential agreement with those previously published. Following partial hepatectomy there was a slight decrease in the

effectiveness of the subcutaneously administered triphenylchloro-
ethylene. A great contrast was noted when the results of intrasplenic
injection were compared in the two groups of animals. There was a
more than three-fold increase in the effectiveness of intrasplenically
administered triphenylchloroethylene after partial hepatectomy.

DISCUSSION

The results presented in the preceding paragraph were entirely
unexpected; indeed partial hepatectomy affected the activation of
triphenylchloroethylene in the opposite direction to that which was
predicted on the basis of our experience with α-estradiol (Segaloff,
1946). Using this identical technique with α-estradiol, we observed

TABLE 1. RESULTS OF ADMINISTRATION OF TRIPHENYLCHLOROETHYLENE BY VARIOUS
ROUTES TO SPAYED AND PARTIALLY HEPATECTOMIZED SPAYED RATS

	Subcutaneous injection			Intrasplenic injection			Ratio SC:IS
	Number of animals	Dose ug.	Estrus %	Number of animals	Dose ug.	Estrus %	
Controls	20	30	25	38	6	37	
	56	40	52	40	7	50	1:0.17
	25	80	80	20	8	90	
Partial hepatectomy	20	40	20	25	1.5	24	
	24	60	50	33	2	49	1:0.03
	20	80	85	24	6	88	

that it was greatly decreased in estrogenic activity by passage through
the liver. Further, it was possible to reduce the degree of hepatic
α-estradiol inactivation strikingly by means of partial hepatectomy.
Accordingly, since triphenylchloroethylene is activated by passage
through the liver, it was our belief that partial hepatectomy would
reduce the activation, but as shown in Table 1, this did not occur.

Naturally, there are many possible explanations of this complex
phenomenon, but the following seems to apply best to our present
knowledge. First, the activation of triphenylchloroethylene is but
one step in its metabolism. It is possible that this step is accomplished
by conversion of the phenyl radical to a phenolic one by the addition
of hydroxyl groups. Such conversions are known to occur, i.e., the
liver is able to convert phenyl-alanine to tyrosine (Bernheim and
Bernheim, 1944). The organism as a whole is also capable of producing
phenolic estrogens from a phenyl "proestrogen" (Stroud, 1940;
Emmens 1943). The second step is then the conjugation and/or oxi-
dation of the estrogen formed in the first step. Therefore, under this
concept, there would be going on two essentially competing processes
which would influence the final estrogenic activity: one to increase it,
the other to decrease it. In order to explain the observed phenomena,
still another assumption is necessary: namely, that the destructive

process (be it oxidation or conjugation) requires more hepatic tissue and is the limiting factor in the final resultant estrogenic activity. Thus partial hepatectomy decreases still further the amount of liver available for the inactivating phase, thereby permitting the activation to predominate and producing the apparently anomalous phenomenon of increasing the activation of triphenylchloroethylene by partial hepatectomy.

SUMMARY

Triphenylchloroethylene was injected subcutaneously and intrasplenically into spayed female rats. The estrogenic response, as judged by vaginal estrus, was increased when the material had to pass through the liver before entering the systemic circulation (intrasplenic injection).

This hepatic "activation" or potentiation of estrogenic activity was increased by partial hepatectomy.

A tentative explanation of these phenomena is presented.

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NOTES AND COMMENTS

METABOLISM OF THE STEROID HORMONES: ANDROS-TERONE ADMINISTRATION TO GUINEA PIGS[1]

In a previous communication it was shown that eight per cent of orally administered androsterone and two per cent of subcutaneously administered androsterone were isolated as isoandrosterone from the urine of normal male guinea pigs (Dorfman, Schiller, and Fish, 1944). The urinary extract from the subcutaneously administered androsterone has been studied further and will be the subject of this report.

EXPERIMENTAL

As reported previously, 80 mg. of androsterone propionate,[2] in 2 cc. of sesame oil, was injected subcutaneously for three successive days into each of six adult male guinea pigs. A total of 1.44 grams of androsterone propionate or 1.2 grams of androsterone was administered. The details of urine collection, extraction, and preparation of the various fractions have been described previously (Dorfman, Schiller, and Fish, 1944). The non-ketonic digitonin precipitable fraction (4.2 mg.) was almost pure cholesterol. The non-ketonic digitonin soluble fraction (89.7 mg.) yielded no pure substances when chromatographed as the acetates using aluminum oxide (Merck).

The ketonic digitonin fraction (109.4 mg.) was subjected to chromatographic analysis as the acetates using 3.3 grams of aluminum oxide (Merck). The column was eluted with carbon tetrachloride, and carbon tetrachloride containing from 0.1 to 1.0 per cent of absolute ethanol. From two elutes containing 0.1 per cent ethanol in carbon tetrachloride there were obtained 3.7 mg. of androsterone (m.p. 179–182°C.) and 5.6 mg. of androsterone (m.p. 178–180°C.), respectively. No depressions in melting points when these samples were mixed with an authentic sample of androsterone. An acetate m.p. 158–160°C. was obtained which when mixed with androsterone acetate (m.p. 160–161°C.) melted at 159–160°C.

SUMMARY

The subcutaneous administration of androsterone to normal male guinea pigs gave rise to isoandrosterone (2 per cent) and androsterone (1 per cent) in the urine.

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A SEX DIFFERENCE IN BLOOD CHOLESTEROL CONTENT IN MICE

In the course of work on the differences in metabolism between mice of different coat colours the whole blood cholesterol has been investigated. The blood cholesterol level is known to rise with unilateral adrenalectomy, and the "normal limits" of this level in man are very wide, usually being given as 150–250 mg. per 100 cc. On the assumption that the adrenals are concerned in physiological pigmentation and the known fact that when the adrenal cortical tissue is damaged (decreased) hyperpigmentation results, the hypothesis for investigation was that mice with dark coat colour (black) should have less cortical tissue than those with light coats (agouti), and therefore might be expected to show a higher blood cholesterol content. No such differences in cholesterol between the two colours of mice could be found, but a marked difference between males and virgin females was noted. This has been investigated.

The mice used were descended from a cross made in 1944 between a male of the CBA (Strong) strain and a female of the C57 black (Little), both of these strains being pure lines. One male and three females, all agouti, resulted from this cross, and the whole of the stock has been developed from these four animals. The succeeding generations segregated for agouti and black coat colour in the ratio of 3:1. For the animals actually used in this work one male was mated to four of his litter sisters, and the blood cholesterol of the offspring determined. The hereditary factors have therefore been kept as constant as possible, bearing in mind the necessity for having mice of two different coat colours.

All the mice were kept under identical conditions of housing and diet, as has the whole colony since its origin. In every case the blood was taken when the mouse was 49 days old, this age being an arbitrary one at which the animal was sufficiently large for exsanguination to be carried out easily. Chloroform anaesthesia was used, the abdomen opened, and the blood removed from the inferior vena cava.

Cholesterol was estimated by the Liebermann-Burchard reaction, the whole blood being extracted with an ether-alcohol mixture. This method of extraction is stated to give high results when compared with the gravimetric digitonide méthod (Reinhold, 1936), but this is not of importance here as it is the relative amounts, not the absolute, which is the essential point. On the other hand estimation of the blue-green colour developed after 15 minutes was found to be insufficiently accurate, this stage in the reaction being too fugitive. The remarks of Snell & Snell (1937) on this point are of interest; "Practically all methods use the blue-green color, probably largely because results can be obtained so promptly." The method finally used was the estimation of the stable yellow colour developed 24 hours after the addition of the sulphuric acid, which has the additional advantage of being more immune to the action of interfering substances (Schube, 1932). The colour was estimated by an EEL photoelectric colorimeter, using a Tricolour Blue light filter. Comparison was with a natural standard developed at the same time as the solution under test. Results are regarded as accurate to within 1% and are given in the table.

Estimation of the significance of these findings has been made by two methods. (1) By the "t" test (Fisher, 1938). This gave a value for "t" of 5.58, n being 36. This value far exceeds the limits of the published tables (Fisher & Yates, 1943), therefore by this method the sex difference is highly significant. (2) A second method was considered to be justified in view of the high degree of uniformity in housing, feeding, age and especially hereditary factors. Because of this uniformity it was considered to be legitimate to regard any male and 'female of these mice as constituting a "pair," in the sense used by Fisher (1938). This makes possible 357 comparisons of the data, though only 38 of these count as independent for the value of n. Estimation of significance by this method shows a mean difference between the sexes of 32.344 ± 3.991, i.e., more than eight times its standard error. The difference

WHOLE BLOOD TOTAL CHOLESTEROL IN MALE AND VIRGIN FEMALE MICE

Litter No.	Cholesterol in mg. per 100 cc.									
	Males					Virgin females				
1	200	200				163	160	149		
2	244	207	196	189	166	166				
3	183					163	160	153	153	134
4	161					146	146	135	132	
5	179	179	170			140	140			
6	164	155	149			149	149	143	143	
7	163	160				143	143			

in cholesterol content between the sexes can therefore be regarded as a real one.

Presumably this difference is due to the male requiring more cholesterol than the female. It appears very doubtful whether this can be explained on the grounds of the male needing more cholesterol than the female for the synthesis of sex hormones. In this respect it is of some interest to record that during the investigations cholesterol in five pregnant females was estimated, giving values of 211, 200, 183, 183 and 176 mg. per 100 cc. These values do not differ significantly from those found for males. It will also be noted that there was a gradual decrease in the cholesterol values found throughout the series, this being more marked in the males than in the females. (The possibility of this being due to changes in the standard solution of cholesterol has been eliminated by repeated comparisons with freshly prepared solutions). It may be associated with parental age, but no definite statement can be made on this point at this stage.

The writer has been unable to find any record of such a difference in other animals. It was at first thought that if this sex difference is also found in man the wide "normal limits" given for blood cholesterol content might be reduced to narrower separate standards for the two sexes, and the clinical usefulness of the estimation increased. However, even under the uniform conditions described the mice showed wide variations. (It should be mentioned here that none of them showed any evidence of macroscopic pathology). When the almost unlimited variations of environmental and hereditary factors in man are considered it appears very doubtful whether any immediate practical application of these findings would be possible, even if they apply to man.

SUMMARY

A sex difference in the whole blood total cholesterol content of black and agouti mice has been demonstrated, the value for males being higher than that for virgin females. This difference is statistically significant.

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16 KETOESTRONE IN THE KOBER REACTION

In 1931, Kober developed a colorimetric method for the determination of estrogenic steroids based upon the finding that a red color is obtained whenever estrone is heated with sulfuric acid, diluted with water, and reheated. Later Kober found the addition of phenol to the sulfuric acid before heating made for a more accurate and sensitive determination. In 1938 he found that the sensitivity of the test could be further improved by substituting 2.5% β-naphthol for the phenol sulfonic acid with other slight modifications.

Other investigators, including Cohen and Marrian (1934), Venning *et al.* (1937), Szego and Samuels (1943), and Cohen and Bates (1947), have developed various chromogenic methods for the estrogenic steroids.

Marrian (1938–39) postulated that the chromogen which is formed in the Kober reaction and which is responsible for the red color is $\Delta^{1,3,5}$ estra-triene-3-ol-16, 17 dione (16 ketoestrone) as the oxonium salt. Marrian based this postulation on the following observations: a) the red colored compound was formed more easily from estriol than from estrone; b) the red color was readily discharged by mild reducing agents; c) the red colored solution deposited an insoluble precipitate when treated with o-phenylenediamine; and d) it is a well known fact that α-diketones and o-quinones yield red colored oxonium salts in acid solution.

16-Ketoestrone was not available to test the validity of Marrian's hypothesis until its synthesis was recently reported by Huffman and Lott (1948). Table 1 compares the chromogenic activity of estrone and estriol with 16-ketoestrone using the modified Kober reaction as well as other

TABLE 1. COMPARISON OF COLOR DEVELOPMENT BY DIFFERENT PROCEDURES

	Estrone		Estriol		16-Ketoestrone	
	After 1st Heating	Final color developed	After 1st heating	Final color developed	After 1st heating	Final color development
Original Kober (1)	bright yellow	rose	yellow	pink	brownish pink	pink tinge (almost colorless)
Modified Kober (2)	blue green (green fluorescence)	orange pink	green (green fluorescence)	yellow orange	pinkish brown	very pale pink
Venning modification (4)	orange (green fluorescence)	deep pink	yellow orange (green fluorescence)	pink	rose	faint pink (almost colorless)
Cohen & Bates modification (6)	green (green fluorescence)	pale pink (green fluorescence)	yellow (green fluorescence)	pale pink (green fluorescence)	very slight brownish pink	yellow tinge (almost colorless)
Sulfuric acid (50% by vol.)	yellow orange (green fluorescence)	pale orange (green fluorescence)	pale brownish pink	colorless	light green (green fluorescence)	pale green

chromogenic procedures. Figure 1 shows the comparative color formation of estrone, estriol and 16-ketoestrone in the Kober reaction using β-naphthol.

EXPERIMENTAL PART

16-Ketoestrone was treated with the Kober reagents in the modified form (1938). The amount in solution was equilibrated with other estrogens and a study made of its absorption of light using the Sheard-Cenco spectrophotometer. The opening slit was set at $\frac{1}{2}$ mm. and the 2 mm. exit slit was used, since light intensity at 508 Mμ allowed the galvanometer to be set at 10. Previous unpublished data shows that the peak of absorption is at 508 Mμ for estrone and at 464 Mμ and 508 Mμ for estriol, using the above procedure and instruments. The concentration was 30γ per ml. The absorption
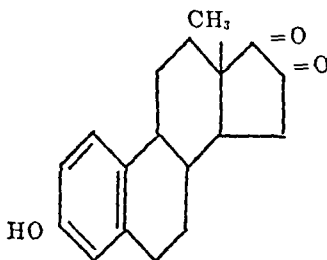
FIG. 1

was made over a range of from 400–580 Mμ. The results shown in Figure 1 is the average of several trial runs at different times on pure material using the modified Kober. All values for any wave length were within experimental error. 16-Ketoestrone was not red in color, showed very little absorption, and reached a doubtful maximal absorption at 508 Mμ or at 540 Mμ depending upon solvent used.

The samples containing 30γ per ml were obtained by diluting accurately a stock of known concentration of the pure substances. Figure 1 records the values for estrone, estriol and 16-ketoestrone when they are dissolved in 95% alcohol, and 16-ketoestrone when dissolved in ether (lower temperature can be used in removing the solvent). One ml of alcohol containing 30γ of each compound was evaporated to dryness in a hot water bath. The Kober reagent was added and heated for 2 minutes at the temperature of boiling water. The tube was then cooled, and 0.6 ml of water was added, the mixtures being kept cool. It was then reheated for $1\frac{1}{2}$ minutes and cooled for 10 seconds. Then 1.8 ml of 65% H$_2$SO$_4$ were added. The blank was 1 ml of 95% alcohol.

DISCUSSION AND CONCLUSIONS

The Kober color reaction with 16-ketoestrone was very faint and the absorption of light was found to be very small when compared to the two estrogens, estrone and estriol (equilibrated quantities). This may mean that this compound is entirely too labile to stand the heating required in the Kober reaction or that it is not chromogenic. It is therefore certainly believed that 16-ketoestrone is not responsible for the color reaction in the Kober test in the method employed in this laboratory. In fact 16-ketoestrone is comparatively colorless in all modifications of the original Kober reaction which was studied.

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THE INABILITY OF THE LIVER TO INACTIVATE THE 3-METHYL ETHER OF BIS-DEHYDRODOISYNOLIC ACID[1]

The 3-methyl ether of bis-dehydrodoisynolic acid (MDDA) has been shown to have estrogenic properties (Tschopp, 1946 and others) although there is lack of agreement regarding its potency as compared with other estrogens (Nicholas, Thayer and Doisey, 1948). Segaloff (1948) has reported that MDDA, unlike most other estrogens, is not inactivated by the liver but rather is potentiated when administered through the hepatic portal system. His conclusions were based upon the finding that the intrasplenic injection of MDDA produced a greater vaginal smear response than did the subcutaneous injection of the compound. Using different criteria and methods we have examined the problem of liver inactivation of this compound under conditions of chronic administration.

MATERIALS AND METHODS

Eighteen male rats weighing approximately 80 grams were divided into three equal groups; a 26 mg. pellet of MDDA was implanted subcutaneously into each animal of the first group, in the second group similar pellets were anchored in a fold of the mesentery, and the third group was kept as normal untreated controls. Similar technique has been used extensively in showing the hepatic inactivation of steroid hormones and other substances. The criterion of effectiveness of the MDDA pellets was the depression of the growth rate which is characteristic action of estrogens in growing male rats. It was thought that growth would be inhibited less in the group having the substance absorbed through the hepatic portal system provided that the liver served as an effective inactivator of MDDA. Between the 24th and the 127th day of treatment animals were killed at intervals for the purpose of examination. At autopsy the pellets were recovered and weighed after drying; various organs were removed, weighed and preserved for histological study.

Absorption of the pellets occured at almost identical rates at the two implantation sites. Approximately half of each pellet had been absorbed by the 45th day and absorption was almost complete at 127 days.

Our results (Figure 1), using growth as a criterion of effectiveness, confirm the idea that absorption through the liver does not decrease the biological potency of MDDA. There was no indication that growth was inhibited less in animals with intramesenteric than with subcutaneous pellets. If there was any difference it was in the other direction but with the number of animals used such differences could not be considered significant. Near the
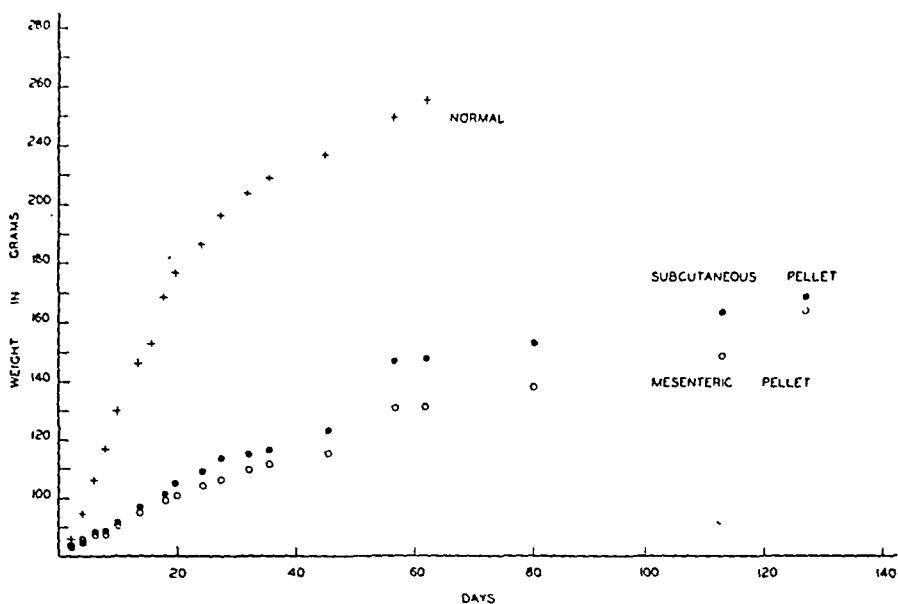
FIG. 1. Showing the mean body weight figures of male rats bearing subcutaneous pellets (solid dots) and mesenteric pellets (open circles) of MDDA.

termination of the observation period the pellets were very small and presumably the amount of MDDA absorbed daily was progressively reduced. This probably accounted for the increased growth rate toward the end of the experiment. Although the amount of MDDA being absorbed was progressively reduced the growth differential exhibited by animals with implants at the two sites was maintained, thus suggesting that dosage level was not the controlling factor.

At autopsy the adrenals and pituitaries were enlarged and the testis and seminal vesicles greatly reduced from normal size as expected from previous reports (Gaunt and Liling, 1947). There was, however, no consistent difference in the size of these organs in the animals having pellets at the two different sites. The effect of more extended chronic treatment on various organs and processes is being studied.

The effectiveness of MDDA pellets in suppressing body growth was not

reduced by implantation on the mesentery as compared with a subcutaneous site. This is interpreted as a confirmation of a previous report that the liver does not inactivate MDDA.

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ASSOCIATION NOTICE
SQUIBB AND CIBA AWARDS AND AYERST, McKENNA AND HARRISON FELLOWSHIP FOR 1948; ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Association for the Study of Internal Secretions has just announced that the award provided by E. R. Squibb and Sons has been conferred on Dr. Fuller Albright of Harvard Medical School, the award furnished by the Ciba Pharmaceutical Company has been made to Dr. Carl G. Heller of the University of Oregon College of Medicine, and the Ayerst, McKenna and Harrison Fellowship has been given to Dr. Ernest M. Brown, Jr., of the George S. Cox Medical Research Institute, University of Pennsylvania.

Doctor Albright, the recipient of the Squibb Award for 1948, has contributed greatly to our understanding of a variety of endocrine functions and dysfunctions in man. His studies on hyperparathyroidism and on calcium and phosphorus metabolism have been incorporated in modern textbooks on these subjects. He has extended this work by making important additions to our knowledge of bone metabolism and its endocrine control, the formation of renal calculi and certain renal disorders. In addition he has described the syndrome of osteitis fibrosa disseminata, pigmentation and endocrine dysfunction which bears his name and he has given us one of the best concepts of Cushing's disease through investigations which applied to this condition the modern ideas of adrenal cortical physiology. Almost every field of endocrine disease has been enriched by his work. Above and beyond this he has been a lucid teacher and a constant inspiration to all his fellow students of endocrinology.

Doctor Albright was born in 1900. He received the degree of Doctor of Medicine from Harvard University in 1924. He served as House Officer at the Massachusetts General Hospital, 1924–26; as assistant medical resident at the Johns Hopkins Hospital, 1927–28 and as Moseley travelling fellow, Harvard, 1928–29. He was instructor and associate in medicine, Harvard Medical School, 1931–39; assistant professor, 1939–42; and associate professor of medicine, 1942 to the present, serving as physician to the Massachusetts General Hospital. He is past president of the American Society for Clinical Investigation and of the Association for the Study of Internal Secretions.

The Ciba Award for 1948 to Doctor Heller was given for his significant contributions to the study of the physiology of reproduction with particular reference to disorders of reproduction in man. His studies have combined techniques of the endocrine laboratory with those of the medical clinic to advance diagnostic and therapeutic procedures in various forms of hypogonadism. Doctor Heller was born in Syracuse, N. Y. January, 1913. He received the degree of Bachelor of Pharmacy in 1935 and the degree of Doctor of Philosophy and Doctor of Medicine in 1940, all from the University of Wisconsin. After interning at the Wisconsin General Hospital he held an American College of Physicians Fellowship in Medicine at Wayne University, 1941–43 and was, successively, instructor in medicine and assistant professor of physiology at Wayne University, 1943–45. Since 1945 he has been associate professor of physiology and medicine at the University of Oregon.

Doctor Ernest M. Brown, Jr. was named to receive the Ayerst, McKenna and Harrison Fellowship for 1948. He was born in 1919 and received the degrees of Bachelor of Arts from West Virginia University in 1941 and of Doctor of Medicine from the University of Pennsylvania in 1944. He served as intern and junior resident at the University of Pennsylvania Hospital 1944–46. Since that time until April 1948 he has been a member of the Army Medical Corps. Doctor Brown will work at the George S. Cox Medical Research Institute with Dr. F. D. W. Lukens on lesions of the islands of Langerhans produced by intravascular infusion of glucose.